Cysteinyl Leukotrienes Dependent $[Ca^{2+}]_i$ Responses to Angiotensin II in Rat Cardiomyocytes and Aortic Smooth Muscle Cells

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ABSTRACT

Angiotensin II (Ang II) plays a very important role in regulating cardiac and vascular contraction and proliferation/hypertrophy via stimulation of AT_1 receptors. A few studies have demonstrated that 5-lipoxygenase (5-LO) derived cysteinyl leukotrienes (CysLT) contribute to Ang II evoked tension responses in rat aortic rings. Whether CysLT would contribute to Ang II evoked Ca^{2+} mobilization in neonatal rat cardiomyocytes (NRC) and rat aortic smooth muscle cells (ASMC) has not been investigated. In the present study, using primary cultures of NRC and minimally passaged cultures of rat ASMC, an effort was made to address this key issue. The agonists evoked increase in cytosolic free calcium ($[Ca^{2+}]_i$) level was determined by fura-2 fluorescence measurement in NRC and ASMC. Total CysLT levels in the culture medium were determined using an ELISA kit. $CysLT_1$/$CysLT_2$ receptor mRNA levels of NRC and ASMC were quantified by Northern blot analysis. In NRC, the AT_1 but not the AT_2 selective antagonist, attenuated the elevations in $[Ca^{2+}]_i$ and CysLT levels evoked by Ang II. Vasopressin (AVP) and endothelin-1 (ET-1) increased $[Ca^{2+}]_i$ but not CysLT levels. The 5-LO inhibitor, AA861, and the $CysLT_1$ selective antagonist, MK-571, reduced the maximal $[Ca^{2+}]_i$ responses (E_{max}) to Ang II but not to AVP and ET-1. While $CysLT_1$ antagonist reduced the E_{max} to leukotriene D_4, (LTD_4), the dual $CysLT_1$/$CysLT_2$ antagonist, BAY u9773, completely blocked the $[Ca^{2+}]_i$ elevation to both LTD_4 and leukotriene C_4 (LTC_4). Both $CysLT_1$ and $CysLT_2$ mRNA were detected in NRC. The inositol 1,4,5 triphosphate

($InsP_3$) antagonist, 2-aminoethoxyphenyl borate (2-APB), attenuated the $[Ca^{2+}]_i$ responses to Ang II and LTD_4. In ASMC, Ang II, ET-1 and AVP evoked $[Ca^{2+}]_i$ responses were significantly higher in the cultured ASMC isolated from spontaneously hypertensive rats (SHR) compared to ASMC derived from age-matched normotensive Wistar-Kyoto (WKY) strain. Addition of either MK571 or BAY u9773, reduced the E_{max} values to Ang II (but not to ET-1and AVP) in both strains. While BAY u9773 abolished the $[Ca^{2+}]_i$ responses evoked by both LTD_4 and LTC_4, MK571, the $CysLT_1$ antagonist reduced the responses evoked by LTD_4 but not LTC_4. The basal CysLT levels were higher in the ASMC of SHR. Ang II but not ET-1 and AVP evoked time and concentration dependent increases in CysLT levels in ASMC of both WKY and SHR strains. The AT_1 selective antagonist, losartan, but not the AT_2 antagonist, PD123319, attenuated the increases in $[Ca^{2+}]_i$ and CysLT levels evoked by Ang II. The $InsP_3$ antagonist, attenuated the $[Ca^{2+}]_i$ responses to Ang II, LTD_4 and LTC_4. Both $CysLT_1$ and $CysLT_2$ mRNA were detected in the ASMC of either strain; but they were significantly higher in SHR. These data suggest that AT_1 mediated CysLT production contributes to Ang II evoked Ca^{2+} mobilization in NRC and that elevated CysLT production along with increased expression of both $CysLT_1$/$CysLT_2$ receptors may account for the exaggerated $[Ca^{2+}]_i$ responses to Ang II in ASMC of SHR due to enhanced mobilization of Ca^{2+} from $InsP_3$ sensitive intracellular Ca^{2+} stores.

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List of Key Abbreviations

AA	Arachidonic Acid
ACE	Angiotensin-Converting Enzyme
ANF	Atrial natriuretic factor
Ang II	Angiotensin II
ASMC	Aortic Smooth Muscle cell
AT_1	Angiotensin Subtype 1 Receptor
AT_2	Angiotensin subtype 2 Receptor
AVP	Arginine vasopressin
B_{max}	Maximal binding capacity
B_0	Maximum binding wells
BP	Blood pressure
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Cytosolic free calcium
CO	Cardiac output
COX	Cyclooxygenase
CR	Concentration-Response
CysLT	Cysteinyl Leukotrienes
$CysLT_1$	CysLT type 1 receptor
$CysLT_2$	CysLT type 2 receptor

DAG	Diacylglycerol
EC_{50}	Concentration required to evoke half maximal response
EGF	Epidermal Growth Factor
EIA	Enzyme Immunoassay
E_{max}	Maximum response
ET-1	Endothelin-1
FAK	Focal adhesion kinase
FLAP	Five lipoxygenase activating protein
Fura-2/AM	Fura-2 acetoxymethylester
HBSS	Hank's balanced salt solution
HETE	Hydroxyeicosatetraenoic acid
HR	Heart rate
HT	Hypertension
$InsP_3$	Inositol 1, 4, 5, triphosphate
LT	Leukotriene
LO	Lipoxygenase
mRNA	Messenger RNA
MAPK	Mitogen-activated protein kinase
NE	Norepinephrine
NO	Nitric Oxide
PGs	Prostaglandins
PGI_2	Prostacyclin

PIP_2	Phophotidyl inositol biphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMNL	Polymorphonuclear leucocytes
SHR	Spontaneously hypertensive rat
SNS	Sympathetic nervous system
SR	Sarcoplasmic reticulum
SRS-A	Slow reacting substance of anaphylaxis
VSM	Vascular smooth muscle
VSMC	Vascular smooth muscle cells
WKY	Wistar-Kyoto strain

Names of Various Inhibitors and Antagonists used in the Study

AA861	5 – LO inhibitor
MK591 (100 nM)	FLAP inhibitor
MK571 (100 nM)	$CysLT_1$ selective antagonist
Bay u9773 (100 nM)	$CysLT_1$/$CysLT_2$ antagonist
2-APB (5 μM)	$InsP_3$ receptor antagonist
Losartan (1 μM)	AT_1 selective antagonist
PD123319 (1 μM)	AT_2 selective antagonist

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1. INTRODUCTION

1.1 The Role of Renin Angiotensin System (RAS) in the Regulation of Cardiovascular function

1.1.1 Generation of Angiotensin II (Ang II)

Ang II plays multiple roles in the regulation of cardiovascular function (Touyz et al, 2000). As an octapeptide hormone, Ang II is the key component of the RAS. It regulates plasma volume, blood pressure, sympathetic nervous system activity, and thirst responses. In addition, Ang II also plays an important role in pathological adaptation, such as myocardial remodeling after myocardial infarction and vascular remodeling in hypertension. The RAS was originally considered as a component of the circulating system. However, later studies have also demonstrated that many of its components are localized in vascular tissues suggesting the existence of a local tissue RAS (Dzau, 1989; Danser, 1996). Thus Ang II can also be generated locally via tissue RAS. In the classical RAS, the circulating renal-derived renin cleaves the hepatic-derived angiotensinogen at the N terminus to generate the decapeptide, angiotensin I. This is subsequently converted in the lungs by the dipeptidyl carboxypeptidase, angiotensin-converting enzyme (ACE) to the active Ang II (Skeggs et al., 1967; Dorer et al., 1972; Phillips et al., 1993). In addition, Ang I can also be converted into the heptapeptide Ang (1-7) by three tissue endopeptidases, neutral endopeptidase (NEP) 24.11, NEP 24.15, and NEP 24.26 (Ferrario et al., 1997). Ang II is metabolized by aminopeptidases to Ang III and Ang IV.

ACE is ubiquitous in distribution as it is present in the cells, plasma as well as in the interstitium. Tissue ACE exists in all major organs including heart, blood vessels, adrenals, kidney, liver, brain, and reproductive organs (Hollenberg, 1998), and it has been shown to be functional in utero (Schutz et al., 1996; Esther et al., 1997). It has been demonstrated that tissue ACE activity reaches the maximum level during the phase of major organ development and gradually decreases thereafter (Esther et al., 1997). Except renin, all other components of the RAS are generated in the cardiovascular system. ACE is observed in high concentrations in vascular adventitia, in cultured vascular smooth muscle cells (VSMC) and in endothelial cells (EC; Dzau, 1989; Ekker et al., 1989; Naftilan, 1994). Angiotensinogen mRNA and protein have been found in cardiac myocytes, vascular smooth muscle (VSM), endothelium, and perivascular fat (Naftilan et al., 1991; Naftilan, 1994; Morgan et al., 1996). As renin is absent in cardiovascular system, local generation of Ang II in the interstitium is controlled by tissue ACE since it is probably dependent on circulating renin. Although the function of tissue ACE currently remains unknown, it may play a very important role in the regulation of regional blood flow as demonstrated in the human forearm, where *in situ* generated Ang II plays a larger role in promoting vasoconstriction than circulating Ang II (Saris et al., 2000).

Ang II also can be generated via non-ACE pathways, and this may have major implications in cardiovascular disease states. Studies have shown that chymotrypsin-like serine protease (chymase) may be an important pathway for the conversion of Ang I to Ang II in the human heart (Urata et al., 1990 & 1996) and kidney (Hollenberg, 1998).

Functional chymase and a non-ACE pathway have also been observed in vascular tissue (Hollenberg et al., 1998; Takai et al., 1998) as well as in the carotid artery (Shiota et al., 1999).

1.1.2 Angiotensin Receptors

The diversity of specific physiological effects evoked by Ang II suggested the existence of multiple type of Ang II receptors (Peach, 1981; Douglas, 1987). The existence of multiple subtypes of Ang II receptors was first confirmed by a pharmacological approach using various specific Ang II receptor antagonists (Chiu et al., 1989). In mammalian cells, Ang II mediates its effects via at least two high-affinity, G-protein coupled plasma membrane receptors, namely AT_1 and AT_2. Both receptor subtypes have been cloned and pharmacologically characterized (Murphy et al., 1991; Sasaki et al., 1991; Kambayashi et al., 1993; Mukoyama et al., 1993). Two other Ang II receptors have been termed, AT_3 and AT_4 subtypes. Since the pharmacology of AT_3 and AT_4 receptors has not been fully characterized, these receptors are not yet included in a definitive classification of mammalian AT receptors as defined by the International Union of Pharmacology Nomenclature Subcommittee for Angiotensin Receptors (de Gasparo, 1995).

A decade ago, the AT_1 receptor was successfully cloned (Murphy et al., 1991; Sasaki et al., 1991). Studies have demonstrated that AT_1 receptor belongs to the seven membrane-spanning G protein-coupled receptor family and typically activates phospholipase C

(PLC) via the heterotrimeric Gq protein coupling (de Gasparo et al., 1995; Inagami, 1995). The human AT_1 receptor gene is located on chromosome 3. The AT_1 receptor is ubiquitously and abundantly presented in adult tissues, including blood vessel, heart, kidney, adrenal gland, liver, brain, and lung. In the vasculature, AT_1 receptors are expressed at high levels in smooth muscle cells and relatively low levels in the adventitia and endothelium (Zhuo et al., 1998; Allen et al., 2000). AT_1 receptor mediates all the classical well known physiological actions of Ang II, such as elevation of blood pressure, vasoconstriction, increase in cardiac contractility, aldosterone release from the adrenal gland, facilitation of catecholamine release from nerve endings, renal sodium and water absorption (Timmermans et al., 1993). This subtype is predominant in the control of Ang II-induced cardiovascular functions (Sadoshima, 1998). In addition, recent accumulating evidence supports the notion that by AT_1 receptor mediated Ang II may contribute directly to the pathogenesis of various cardiovascular and renal diseases (Kim and Iwao, 2000). In rodents, two AT_1 receptor subtypes have been identified, namely AT_{1A} and $AT_{1B,}$ which have 94% homology with regard to amino acid sequence and have similar pharmacological properties and tissue distribution patterns (Iwai and Inagami, 1992; Kim and Iwao, 2000). AT_{1A} and AT_{1B} receptor genes in rats are located in chromosome 17 and 2, respectively. Studies have revealed that AT_{1A} is the dominant subtype expressed in the liver, kidney, vasculature, lung, ovary, and heart, whereas AT_{1B} is expressed in greater quantities in the adrenal, anterior pituitary, and uterus (Sandberg et al., 1992;

Kakar et al., 1992; Curnow et al., 1992; Iwai and Inagami, 1992). The expression of these receptors in the brain varies from region to region. The AT_1 receptor is a glycoprotein and consists of 359 amino acids (Sandberg, 1994). It contains extracellular glycosylation sites at the amino terminus (Asn4) and the second extracellular loop (Asp176 and Asn188) (Desarnaud et al., 1993). The transmembrane domain at the amino-terminal extension and segments in the first and third intracellular loops are responsible for G protein interactions with the receptor (Hjorth et al., 1994). Internalization of G protein-coupled receptors involves receptor phosphorylation, which may be partially mediated by caveola (Berk and Corson, 1997; Ishizaka et al., 1998). Though G protein-coupled receptors do not have intrinsic kinase activity, they are phosphorylated on serine and threonine residues by the G protein receptor kinase (GRK) family members. AT_1 receptors are phosphorylated both in the basal state and in response to Ang II stimulation (Kai et al., 1994). Threonine and serine residues between Thr332 and Ser338 of the cytoplasmic tail are fundamental for receptor internalization (Hunyady et al., 1994). In addition, the AT_1 receptor is also phosphorylated at tyrosine residues. Possible tyrosine phosphorylation sites within the AT_1 receptor include amino acids 302, 312, 319, and 339 within the carboxyl terminus (Berk and Corson, 1997). Tyrosine at position 319 is very important as it is part of the motif Tyr-Ile-Pro-Pro, which is analogous to a Src homology 2 (SH2) binding motif in the PDGF receptor (Tyr-Ile-Pro) and in the epidermal growth factor (EGF) receptor (Tyr-Leu-Pro-Pro) (Fantl et al., 1993). In EGF and PDGF receptors, these motifs are target sequences for tyrosine phosphorylation. Various

tyrosine kinases, including Src family kinases, Janus kinases (JAK and TYK), and focal adhesion kinase (FAK) can phosphorylate tyrosine residues on AT_1 receptors.

The other major isoform of Ang II receptor is AT_2 subtype. The AT_2 receptor gene is localized as a single copy on the X chromosome. Distribution of AT_2 binding sites was studied using autohistoradiography of nonspecific radioactive Ang II ligands followed by displacement with specific antagonists. AT_2 is usually ubiquitously expressed in fetal tissues and declines rapidly after birth, suggesting a possible role of this receptor in fetal development and organ morphogenesis (Nahmias and Strosberg, 1995). In adults, AT_2 receptor expression is detectable in the heart, pancreas, kidney, brain, adrenals, and vasculature (Viswanathan and Saavedra, 1994; Touyz et al., 1999a). In the vasculature, AT_2 receptors predominate in the adventitia tissue and are detectable in the media (Zhuo et al., 1998). AT_2 receptors are also expressed in several cell lines (Inagami, 1995). Unlike the AT_1 receptor, there is no evidence for subtypes of the AT_2 receptor. The AT_2 receptor is also a seven transmembrane domain. It is a G_i protein-coupled receptor, comprising 363 amino acids. It has low amino acid sequence homology (~32%) with AT_{1A} or AT_{1B} receptors (Mukoyama et al., 1993). Although the exact signaling pathways recruited by AT_2 receptor activation and its functonal role remains still elusive, AT_2 subtype receptor seems to be regulated by intracellular cations, particularly Na^+

(Tamura et al., 1999). AT_2 activation may antagonize, under physiological conditions, AT_1-mediated effects (Ciuffo et al., 1998; Yamada et al., 1998) by inhibiting cell growth, and by promoting apoptosis and vasodilation (Hayashida et al., 1996; Horiuchi, 1997a,b; Gallinat et al., 2000; Unger, 1999; Siragy, 2000). In contrast to extensive data on the molecular and cellular functions and pathophysiological significance of AT_1 receptor, the role of AT_2 receptor in cardiovascular diseases remains to be defined.

1.1.3 Actions of Ang II on Cultured Cardiac Myocytes

Ang II exerts effects directly and indirectly on the cardiovascular system (Rogers and Lokuta, 1994). It directly stimulates cardiomyocyte excitation-contraction coupling, causing positive inotropic and chronotropic responses in cardiac muscle (Schomisch, 1990; Ishihata, 1993), and indirectly influences these parameters by facilitating adrenergic neurotransmission (Blumberg, 1975). In addition to these physiological actions, Ang II has been associated with pathological consequences such as myocardial ischemia, hypertrophy, and pressure overload (Morgan, 1991; Dostal, 1993). Although cardiac myocytes express both AT_1 and AT_2 receptors (Booz and Baker, 1996), almost all of the biological responses to Ang II reported so far are mediated by AT_1 receptor. Accumulating evidence has established that Ang II causes hypertrophy of neonatal cardiac myocytes (Baker and Aceto, 1990; Baker et al., 1992; Sadoshima and Izumo, 1993 & 1997) and adult myocytes (Wada et al., 1996; Liu et al., 1998; Ritchie et al., 1998). In NRC, Ang II directly induced the fetal phenotype of gene expressions, such as

those of β-MHC, skeletal α-actin, and ANF, indicating the direct involvement of AT_1 receptors in cardiac gene reprogramming *in vitro* (Sadoshima and Izumo, 1993). Furthermore, Ang II stimulated the expression of immediate-early genes, including c-fos, c-jun, jun B, Egr-1, and c-myc (Sadoshima and Izumo, 1993). However, the significance of Ang II induced immediate-early genes expression in hypertrophy is unclear.

Ang II, via AT_1 receptor, activates a diversity of intracellular signaling cascades in NRC, although the role of such signaling cascades in myocyte hypertrophy or gene reprogramming remains to be elucidated. Cardiac myocyte AT_1 receptor couples to a heterotrimeric G protein, stimulates the activation of phospholiase C (PLC), and causes increases in inositol 1,4,5 trisphosphate ($InsP_3$) and diacylglycerol (DAG). This in turn leads to an increase in the release of Ca^{2+} from intracellular stores and activation of protein kinase C (PKC). In addition, it has been reported that Ang II activates a variety of kinases (Sadoshima et al., 1995; Kudoh et al., 1997; Takano et al., 1996; Sadoshima and Izumo, 1995), phospholipases A_2 (PLA_2) and D (PLD) resulting in increased generation of phosphatidic acid and arachidonic acid (Sadoshima and Izumo, 1997). Ang II also induces phosphorylation of various kinases, signal transducers (Kodama et al., 1998). However, it remains unclear whether Ang II can indeed simultaneously activate all of the above-mentioned signaling cascades in myocytes. Furthermore, it remains to be determined to what extent each signaling cascade is involved in Ang II-induced cardiac

myocyte hypertrophy and gene reprogramming seen in pathological cardiac hypertrophy states encountered *in vivo*.

1.1.4 Ang II Evoked Ca^{2+} Signaling in Cultured NRC

The presence of all components of the RAS in neonatal rat cardiomyocytes (NRC) is consistent with its role in maintaining cardiovascular homeostasis (Booz and Baker, 1996; Touyz et al., 1996a). Ang II regulates cardiac contractility and growth via stimulation of AT_1 receptors (Booz and Baker, 1996; Touyz et al., 1996a). The multiple cardiac actions of Ang II are mediated by changes in $[Ca^{2+}]_i$ (Rogers and Lokuta, 1994). Ang II activates inwardly directed Ca^{2+} currents (Allen et al., 1988), giving rise to increased Ca^{2+}-induced Ca^{2+} release from cardiac sarcoplasmic reticular stores (Kem et al.,1991). Stimulation of AT_1 receptors leads to Ca^{2+} mobilization through the activation of PLC, resulting in the generation of $InsP_3$ and DAG (Kem et al., 1991; Sadoshima et al., 1993a; Touyz et al. , 1996a & 2000). The mechanisms governing the regulation of Ang II evoked increases in cystosolic free calcium ($[Ca^{2+}]_i$) level are not fully understood. Besides enhancing the $[Ca^{2+}]_i$ level, Ang II elicits complex intracellular signaling events that include the production of superoxide anions, the activation of several kinases as well as the alteration of cyclic nucleotides and nitric oxide (NO) levels (Touyz et al., 2000). In addition, ANG II activates phospholipase (PLA_2) and phospholipase D (PLD), resulting in elevation of arachidonic acid (AA)-derived

metabolites (Dulin et al., 1998; Lokuta et al., 1994; Nasjletti et al., 1998; Parmentier et al., 2001; Rao et al., 1994; Zafari et al., 1999).

1.1.5 Vascular Actions of Angiotensin II

Ang II mediates its effects directly by acting on Ang II receptors located on vascular cells, indirectly via the release of other factors, and possibly via cross-talk with intracellular signaling pathways of other vasoactive substances and growth factors. Except the main function of vasoconstriction, vascular smooth muscle cells have important synthetic properties during development and vascular remodeling and are the major source of extracellular matrix components of the vascular media (Katoh and Periasamy, 1996). During blood vessel development, immature smooth muscle cells are in a dynamic state of growth and differentiation characterized by proliferation and migration (Glukhova et al., 1991). While in the adult vessel, the smooth muscle cells are quiescent with a fibroblast-like appearance, and are filled with contractile fibers (Gordon et al., 1990). Although mature smooth muscle cells remain quiescent until injury or insult occurs, they undergo physiological hypertrophy in response to increased load (Bucher et al., 1982; Katoh and Periasamy, 1996). Ang II plays a role in these developmental processes, acting via AT_1 and AT_2 receptors, which are differentially expressed in vascular smooth muscle cells (VSMC) during normal development and during pathological processes. In cardiovascular disease states, smooth muscle cells undergo hyperplasia and/or hypertrophy as an adaptive or reactive response (Geisterfer et al., 1988; Berk et al., 1989; Paquet et al., 1990; Stouffer and Owens, 1992; Dubey, 1997; Touyz and Schiffrin, 1997a; Touyz et al., 1999b). This may be critical in vascular remodeling associated with hypertension, atherosclerosis, or neointimal formation. Ang

II has been established to stimulate protein synthesis and induce cellular hypertrophy in cultured VSMC via activation of AT_1 receptors (Geisterfer et al., 1988; Berk et al., 1989). This pathway is considered to contribute in a major way towards the development of various vascular diseases including hypertension and atherosclerosis (Gibbons and Dzau, 1994). A growing body of evidence shows that AT_1 receptor activation in cultured VSMC, coupled to the G protein Gq, causes not only activation of PLC-β leading to increases in DAG and $[Ca^{2+}]_i$ but also activation of multiple signal transduction cascades.

While Ang II evokes vasoconstriction, growth, migration, production of extracellular matrix components, and inflammation via AT_1 receptors, it promotes apoptosis, and attenuates proliferation and hypertrophy via AT_2 receptors activation (Allen et al., 2000; Siragy, 2000). In addition, the bioactive end products of the RAS, such as Ang-(1-7), may also modulate vascular responses to Ang II. As a naturally occurring competitive inhibitor of Ang II, Ang-(1-7) is a potent vasodepressor and has antihypertensive effects. It can increase the release of vasopressin, stimulate synthesis and release of vasodilator prostaglandins, potentiate the actions of bradykinin and release nitric oxide (Ferrario et al., 1997). The receptor mediating the vascular actions of Ang-(1-7) has been described as a non-AT_1/AT_2 subtype (Ferrario et al., 1997). Although the precise role of this peptide in the physiological and pathophysiological regulation of vascular function

remains unclear, its potential to antagonize AT_1-mediated actions suggests that Ang-(1-7) may be involved in the modulation of vascular tone by promoting vasodilation.

1.1.6 Ang II evoked Ca^{2+} Signaling in Blood Vessels

Ang II plays an important role in both the physiological maintenance of arterial pressure and the pathogenesis of hypertension by regulating the vasoconstriction and growth via multiple intracellular signaling pathways that employ $[Ca^{2+}]_i$ as a second messenger (Touyz et al., 2000). $[Ca^{2+}]_i$ is a fundamental regulator of VSMC contraction and proliferation (Clapham et al., 1995). Most of the vascular actions of Ang II are mediated predominantly by activation of the AT_1 subtype (Touyz et al., 2000). Stimulation of AT_1 receptors leads to Ca^{2+} mobilization through the activation of PLC, resulting in the generation of $InsP_3$ and DAG (Kem et al., 1991; Sadoshima and Izumo, 1993a; Touyz et al., 1996a & 2000). Ang II-induced exaggerated $[Ca^{2+}]_i$ response leads to vasoconstriction, vascular hypertrophy and hypertension in SHR (Touyz et al., 1997b). The mechanisms underlining the regulation of Ang II evoked increases in $[Ca^{2+}]_i$ level are not fully understood.

1.1.7 Ang II Evoked Increase in Intracellular Free Calcium Concentration

Ang II-stimulated Ca^{2+} signaling is complex and occurs via multiple pathways to elicit an integrated Ca^{2+} signal. Ang II typically mediates a biphasic $[Ca^{2+}]_i$ response comprising a rapid initial transient phase and a sustained plateau phase (Dostal, 1990; Touyz et al., 1994; Assender et al., 1997; Touyz and Schiffrin, 1997b). Both AT_{1A} and AT_{1B} receptors have been shown to mediate calcium signaling in rodent VSMC (Zhu et al., 1998b). The first $[Ca^{2+}]_i$ transient is generated primarily by $InSP_3$-induced mobilization of intracellular Ca^{2+} and to a lesser extent by Ca^{2+}-induced Ca^{2+} release (Touyz and Schiffrin, 1997b). The second $[Ca^{2+}]_i$ phase, which appears to contribute to the sustained Ang II-induced vasoconstriction, is dependent on external Ca^{2+} and is the result of transmembrane Ca^{2+} influx (Rembold, 1992; Ruan and Arendshorst, 1996a; Inscho et al., 1997; Iverson and Arendshorst, 1998; Touyz et al., 1999c). The exact mechanism that contributes to Ang II stimulated Ca^{2+} influx is unclear but it may involve voltage-dependent calcium channels, Ca^{2+}-permeable, nonspecific dihydropyridine-insensitive cation channels, receptor-gated Ca^{2+} channels, Ca^{2+}-activated Ca^{2+} release channels, and activation of the Na^{+}/Ca^{2+} exchanger (Arnaudeau et al., 1996; Lu et al., 1996). In addition to $InSP_3$-mediated mobilization of intracellular Ca^{2+} and influx of

extracellular Ca^{2+}, tyrosine kinase-dependent increases in $[Ca^{2+}]_i$ have been demonstrated in VSMC (Hughes and Bolton, 1995; Touyz et al., 1996a; Di Salvo et al., 1998).

1.2 Leukotrienes

1.2.1 Introduction

Leukotrienes (LTs) are a family of lipid mediators involved in acute and chronic inflammatory and allergic diseases (Samuelsson, et al., 1983). They are 5-lipoxygenase (5-LO) derived metabolites of AA. LTs are normal products of continuous metabolism of AA and present in many cells and organs. While little is known about the effects of LTs on physiological functions, studies have shown that LTs play an important role in the mediation of several pathophysiological processes such as generalized or local immune reactions, inflammation, asthma, shock, and trauma. LTs exert effects on many essential organs and systems, including the cardiovascular system, the pulmonary system, the central nervous system, the gastrointestinal tract, and the immune system. In these organs, the effects of LTs are mediated by specific LT receptors. Therefore, identification of LTs and characterization of their systemic and local pathological effects, together with characterization of their structure-activity relationships, are fundamental to developing LT antagonists or synthesis inhibitors that might prevent or reverse LT-dependent reactions (Feuerstein and Hallenbeck, 1987).

1.2. 2 Discovery of Leukotrienes

It is well known that there are important interactions between the AA and its cyclooxygenase (COX) products, and the anaphylactic mediator, slow-reacting substance of anaphylaxis (SRS-A) and the nonimmunologically generated SRSs that promote bronchospasm. COX inhibitors such as aspirin or indomethacin attenuated the production of prostaglandins (PGs) from human or guinea pig lung during antigen challenge but greatly increased the generation of SRS-A. The generation of SRS-A and SRS was increased by the addition of fatty acid substrates for lipoxygenase (LO), suggesting that the SRSs are the product of a LO (Piper, 1984). Samuelsson et al first observed that AA is oxygenated at C-5 in a study of the transformation of polyunsaturated fatty acid in rabbit polymorphonuclear leukocytes (PMNL) in 1976. Subsequently a number of derivatives, including Leukotriene B_4 (LTB_4) were identified by the same group. Extension of these studies in 1979 led to the discovery of the pivotal epoxide intermediate, 5(S)-trans-7, 9-trans-11,14-cis-eicosatetrenoic acid (leukotriene A4, LTA_4) and the elucidation of the structure of SRS-A as a group of cysteinyl LTs (CysLTs), namely LTC_4, LTD_4 and LTE_4 (Feuerstein, 1984; Samuelsson, 2000).

1.2.3 Biosynthesis of Leukotrienes

Originally, biosynthesis of LTs has been observed in a variety of cells including PMNL (Hansson and Radmark, 1980), macrophages (Rouzer et al., 1980), mast cells (Razin et al., 1982) and eosinophils (Jorg et al., 1982). In addition, LTs are also found in lungs (Morris et al., 1980), heart (Evers et al, 1985), and brain (Lindgren et al., 1984). More

recently, several lines of evidence also support the generation of LTs in blood vessels (Sjostrom et al., 2001; Maclouf et al., 1989; Samuelsson, 1983; Piomelli et al., 1987).

LT is generated from the AA in the present of activated 5-LO. This enzyme uses AA as substrate and directs the formation of the highly unstable allylic epoxide LTA_4, which then becomes a substrate, depending on the cellular environment and the availability of the enzymes, for either a specific LTA_4 hydrolase enzyme or a LTC_4 synthase enzyme. The LTA_4 hydrolase expressed in neutrophils acts to add a water molecule to the epoxide leading to the generation of a chemically stable and steriospecific product known as LTB_4. LTC_4 synthase is expressed in various types of hematopoietic cells such as mast cells, basophils, eosinophils and monocytes/macrophages. Recent study has shown that LTC_4 synthase is also present in human umbilical vein endothelial cells (Sjostrom et al., 2001). The function of LTC_4 synthase is to add a glutathione moiety to LTA_4 resulting in the creation of the "peptide" LTs. The peptide LTs comprises the sequential products- LTC_4, LTD_4 and LTE_4. LTD_4 and LTE_4 are the result of a successive cleavage of amino acids (**Figure 1**). These three peptide LTs, namely CysLTs (**CysLT**), individually and collectively account for the potent biological activities of the SRS-A are classically considered as inflammatory mediators. Beside 5-LO enzyme, there are other LO enzymes, which when stimulated oxidize AA at the 12 and 15 positions resulting in the formation of 12 HETE and 15 HETE respectively (Letts et al., 1987).

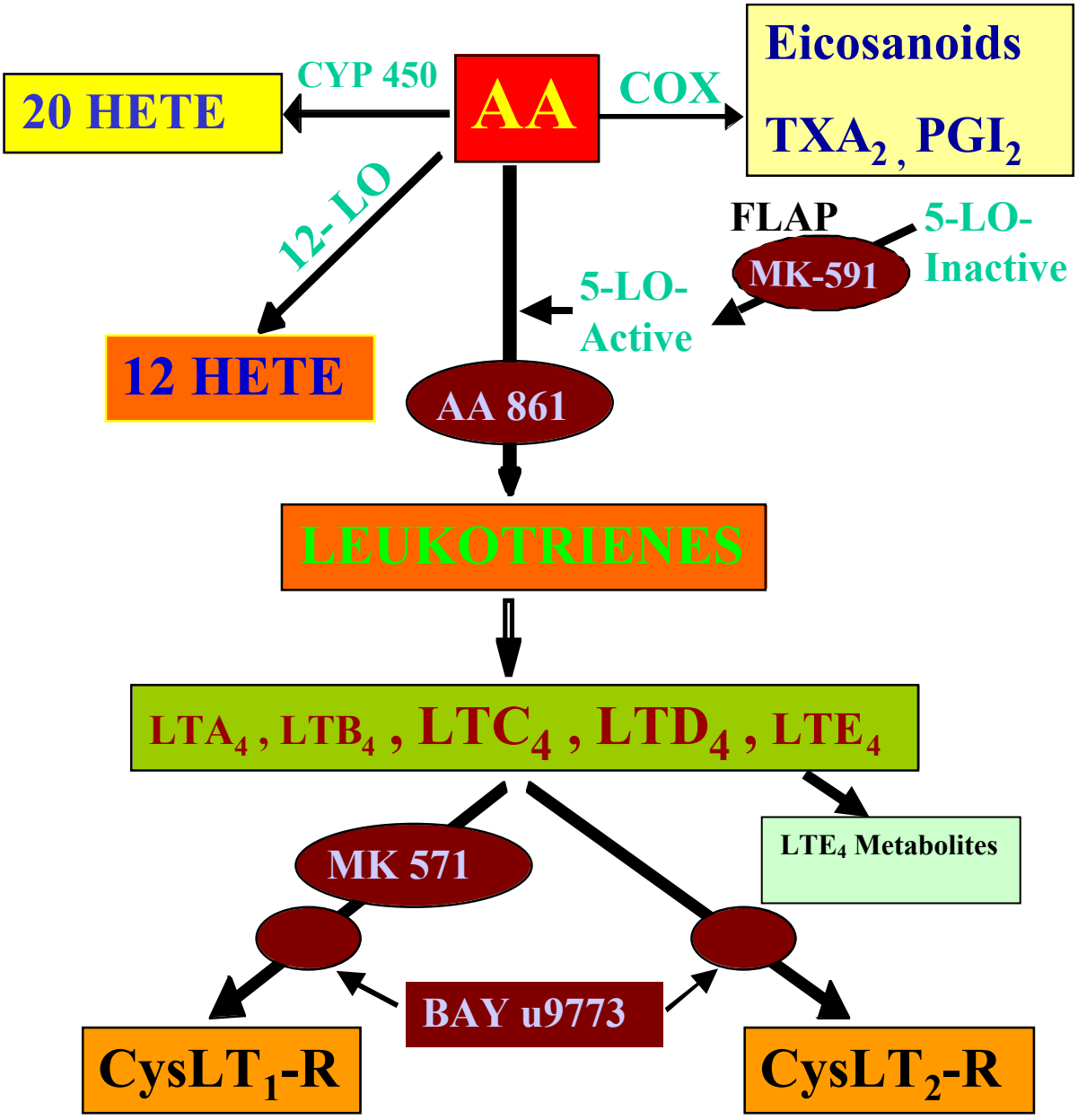


Figure 1. Schematic representation of the biosynthetic pathways of 5-lipoxygenase derived arachidonic acid (AA) metabolites. The site of action of 5-LO inhibitors and CysLT antagonists are shown. **Note:** AA861 is a selective 5-LO inhibitor. MK591 is a 5-LO activating protein inhibitor (FLAP inhibitor) that inhibits activation of 5-LO. MK571 is a $CysLT_1$ selective antagonist whereas Bay u9773 is a dual $CysLT_1$/ $CysLT_2$ receptor antagonist.

1.2.4 5-LO and 5-LO Activating Protein

1.2.4.1 5-LO and its Inhibitors

5-LO has been purified from different types of leukocytes, as monomeric enzymes with molecular weights estimated to be between 72,000 and 80,000. It was abundantly present in monocytes/macrophages, dendritic cells, mast cells and neutrophilic granulocyte. Importantly, 5-LO is also present in the vasculature. The increased expression of 5-LO in pulmonary artery endothelial cells has been observed in disease status such as pulmonary hypertension and challenge. (Spanbroek et al., 2003; Soberman et al., 1985; Zhang et al., 2002). The 5-LO pathway was abundantly expressed in arterial walls of patients afflicted with various lesion stages of atherosclerosis of the aorta, coronary, and carotid arteries. In addition, the number of 5-LO activity markedly increased in cells in advanced lesions (Spanbroek et al., 2003). 5-LO has a central role in LT biosynthesis. It catalyzes the conversion of AA to 5(S)-hydroperoxy-6trans-8,11,14-cis-eicosatetraenoic acid, and further to LTA_4. The activity of 5-LO is Ca^{2+} dependent; moreover, adenosine triphosphate, lipid hydroperoxide, cytokines and other nucleotides enhance 5-LO activity in a Ca^{2+} dependent manner. In the resting cell, 5-LO is present in the cytosol. When 5-LO is activated in the presence of Ca^{2+}, 5-LO from the cytosol is translocated to the nuclear membrane to promote LT biosynthesis (Yamamoto et al., 1984; Radmark et al., 2000).

Several compounds have been shown to inhibit 5-LO, thus preventing LT biosynthesis. AA-861, a 5-LO inhibitor, reduced the synthesis of the SRS-S by lung fragments of

sensitized guinaea pigs. While AA861 also inhibited other LOs like 12-LO, however the IC_{50} value for inhibiting 12-LO were higher by two orders of magnitude. Thus, AA-861 was a relatively selective inhibitor of 5-LO at concentration range (<25 μM). Although compounds such as BW755C, nordihydroguaiaretic acid, 15-Hydroxy-5,8,11,13-eicosatetreenoic acid, were effective inhibitors of 5-LO, they were non-specific and less potent in comparison to AA–861. Thus, among 5-LO inhibitors, AA861, appears to serve as an useful biochemical and pharmacological tool to selectively block 5-LO mediated generation of LT and CysLTs (Yoshimoto et al., 1982). The site of action of AA861 in AA-derived metabolites is shown earlier (**Figure 1,** see page 18).

1.2.4.2 5-LO-Activating Protein (FLAP) and FLAP Inhibitors

The 5-LO activating protein (FLAP) is a novel 18-Kda leukocyte membrane protein with three transmembrane-spanning regions and two hydrophilic loops, functions as a membrane anchor for 5-LO. Both 5-LO and FLAP are necessary for cellular LT synthesis. FLAP is essential for the activation of 5-LO. It was suggested that LT biosynthesis can be a two step process consisting of FLAP-independent binding of 5-LO to the membrane of the nuclear envelope, followed by FLAP-dependent activation of the enzyme (Brideau et al., 1992; Radmark, 2000). Studies regarding the subcellular localization have demonstrated that most of the FLAP is associated with the nuclear membrane, and some with endoplasmic reticulum (Peters-Golden et al., 1993).

Biosynthesis of LTs can be attenuated by employing FLAP inhibitors. MK886 is a potent and specific inhibitor of leukotriene biosynthesis *in vivo* and in intact cells. While

MK886 has no direct effect on 5-LO activity in cell-free systems, it exhibited high affinity for binding to FLAP (Gillard et al., 1989; Rouzer et al., 1990). Inclusion of MK886 led to blockade of 5-LO by preventing the translocation of 5-LO from the cytosol and the subsequent activation of 5-lipoxygenase (Rouzer et al., 1990). MK591 is a structural analogue of MK886, differing in the nature of the indolyl-5-substituent. It also inhibited 5-LO translocation and LT biosynthesis by a specific interaction with FLAP. MK591 is currently undergoing clinical evaluation as a potential agent for the treatment of asthma and inflammatory bowel disease. MK591 may have advantages over MK886, including increased bioavailability and increased activity, as reflected by an approximately six fold increase in potency in the human whole blood assay (Brideau et al., 1992; Prasit et al., 1993).

1.2.5 CysLT Receptors

The CysLTs exert their biological actions by binding and activating at least two distinct but related 7-transmembrane domain, G-protein coupled receptors, namely $CysLT_1$ receptor ($CysLT_1$-R) and $CysLT_2$ receptor ($CysLT_2$-R). Both receptors are linked to PLC activation and Ca^{2+} mobilization. These receptors have been cloned, characterized; they are found to be localized to human lung smooth muscle, peripheral leukocytes, human embryonic kidney, rat vascular sooth muscle cells and heart cells (Lynch et al., 1999; Sarau et al., 1999; Heise et al., 2000; Figueroa et al., 2001; Kamohara et al., 2001; Martin et al., 2001; Mazzetti et al, BJP, 2003). The potency characteristics of LTD_4, LTC_4 and LTE_4 and synthetic antagonists have been elucidated (Lynch et al., 1999; Heise et al.,

2000). Affinity characteristics of various ligands at $CysLT_1$ and $CysLT_2$ are: $CysLT_1$: LTD_4 > montelukast > pranlukast > MK571 > LTC_4=LTE_4= Bay u9773 >>LTB_4. $CysLT_2$: LTC_4 = LTD_4 > LTE_4 = Bay u9973 > MK571 > pranlukast > montelukast.

1.2.5.1 Characterization of $CysLT_1$ Receptor

The complementary DNA (cDNA) for the $CysLT_1$ receptor has been recently cloned, and encodes a 337-amino acid, G-protein-coupled receptor (GPCR) putatively having a 7-transmembrane spanning domain (Lynch et al., 1999; Sarau et al., 1999). The major intracellular signaling pathway for activation of the recombinant $CysLT_1$ receptor is linked to calcium release. Studies have shown the presence of $CysLT_1$-R mRNA in human airway smooth muscle, alveolar macrophages, peripheral blood monocytes, eosinophils (Lynch et al., 1999; Figueroa et al., 2001), EC (Sjostrom et al., 2001), VSMC (Mazzetti et al., 2003) and human embryonic kidney cells (Sarau et al., 1999). The rank order of affinities of the CysLTs for the $CysLT_1$-R defined with transfected cells is: LTD_4>>LTC_4>LTE_4>>LTB_4 (Maekawa et al., 2002). Another group has ranked the affinities of various CysLTs interacting at $CysLT_1$-R as follows: LTD_4>>LTC_4=LTE_4>>LTB_4. The affinity of LTE_4 for the $CysLT_1$-R was approximately 200 fold lower than that of LTD_4 (Lynch et al., 1999).

Although there is circumstantial evidence supporting the hypothesis that CysLT plays an important role in asthma, definitive proof requires clinical evaluation of potent and selective LTD_4 receptor antagonists in the management of bronchial asthma. L-660,711 (MK 571) is an extremely potent and highly selective LTD_4 (but not LTC_4) receptor antagonist on guinea pig ileum and human airway smooth muscle. Receptor binding assays and a variety of quantitative pharmacological studies have shown that MK571 directly interacts with the LTD_4 receptor to produce its pharmacological effects. MK 571 has a unique profile of activity, which represents a substantial improvement over first generation LTD_4 antagonists such as the nonspecific antagonists FPL-55712 and LY-171883 as well as the more functionally specific antagonists, L-649,923 and L-648,051. MK 571 has comparable affinity characteristics to two recently described high affinity LTD_4 receptor antagonists, SKF-104,353 and ICI-198,615. Moreover, it is 100 times more potent than most first generation compounds at inhibiting LTD_4 binding and contractile effects in both guinea pig and human bronchial smooth muscles. The pharmacological profile (high affinity binding to $CysLT_1$-R, oral bioavailability, and long duration of action) indicates that this compound has the potential to be clinically efficacious in disease where LTD_4 receptors are activated (Jones et al., 1989).

The current agents in this class of compounds that have clinical relevance in the management of bronchial asthma are montelukast, zafirlukast, pranlukast and probilukast. They are potent competitors for the binding of radiolabelled LTD_4 to the $CysLT_1$-R with

the following rank order of potency: pranlukast = zafirlukast > montelukast > probilukast (Sarau et al., 1999). The localization of the $CysLT_1$-R in smooth muscle and alveolar macrophages correlates well with the antibronchoconstrictive and anti-inflammatory nature of these new therapeutics entities (Lynch et al., 1999; Martin et al, 2001; Ferreira et al., 2001).

1.2.5.2 Characterization of $CysLT_2$ Receptor

The human $CysLT_2$ receptor was localized to chromosome 13q14 by radiation hybrid and somatic cell hybrid analyses. Like $CysLT_1$receptor, $CysLT_2$ receptor is a putative seven transmembrane spanning domain G protein-coupled receptor. $CysLT_2$ receptors are expressed on alveolar macrophages, airway smooth muscle, cardiac purkenjie cells, adrenal medullary cells and peripheral blood leukocytes and brain cells (Heise et al., 2000). $CysLT_2$-R mRNA levels are detected at high levels in the human atrium and ventricle and at intermediate levels in the coronary artery (Kamohara et al., 2001). Further analysis *by in situ* hybridization revealed that $CysLT_2$-R mRNA is expressed in myocytes, fibroblasts and VSMC (Kamohara et al., 2001). At $CysLT_2$-R subtype, the agonist potency rank order is: LTC_4=LTD_4>>LTE_4>>LTB_4 (Heise et al., 2000; Nothacker et al., 2000; Maekawa et al., 2002). The expected affinity of LTD_4 for $CysLT_2$-R was approximately 50-fold lower than that of LTC_4 in radioligand binding studies, while LTC_4 and LTD_4 induced Ca^{2+} mobilization with equipotency (Heise et al.,

2000; Kamohara et al., 2001). It has been suggested that LTC_4 could promote the development of atherosclerosis as a chemoattractant and exacerbate the disease as a contractile factor of the coronary artery. This is proposed to be mediated via activation of $CysLT_2$-R on VSMC (Kamohara et al, 2001).

Further analysis using $CysLT_2$-R agonist/antagonists would lead to the elucidation of the pathophysiological roles of CysLTs in the cardiovascular system. Bay u9773, is reported to function as a dual $CysLT_1$/$CysLT_2$ antagonist (Heise et al., 2000). However, when Bay u9773 was administered alone, it activated $CysLT_2$-R more potently than LTE_4, but less potently than LTC_4 or LTD_4. Therefore, Bay u9773 is suggested as a selective agonist for the $CysLT_2$ and that it could be a partial agonist. Thus, Bay u9773 could be used as a selective tool for examining the physiological roles of the $CysLT_2$ receptor in cardiac, neuronal, endocrine, and inflammatory circuits (Nothacker et al., 2000). However, the identification and relative distributions of vascular $CysLT_1$-R and $CysLT_2$-R and their regulation in pathological states have not been characterized.

1.2.6 Leukotrienes in Health and Disease

Peptide LTs have potent biological actions on a variety of smooth muscle preparations, whereas LTB_4 is a chemokinetic and chemotactic agent that has relatively few smooth muscle stimulating actions (Piper, 1984).

1.2.6.1 Leukotrienes and Pulmonary System

The lungs of both humans and experimental animals have been shown to produce SRS-A and LTs subsequent to immune and non-immune stimuli. The LT receptors have been characterized in lung parenchyma, bronchial tree, and pulmonary vessels (Piper, 1984; Feuerstein and Hallenbeck, 1987). Among LTs, LTC_4 and LTD_4 are about equally active in contracting isolated bronchi and pulmonary parenchymal strips from humans and experimental animals. Patients with asthma are especially sensitive to these actions of LTs. Furthermore, LTs promote mucous production from bronchial epithelial cells and decrease ciliary activity, both of which contribute to further airway obstruction in asthmatic patients (Feuerstein and Hallenbeck 1987; Henderson et al, 2002). The potential role of LTs in mediating asthma is further supported by recent demonstration that a selective CysLT antagonist ameliorated the attacks in asthmatic patients (Centanni and Santus, 2002).

1.2.6.2 Cys LT- Paradoxical Effects in Vascular Preparations

In addition to their bronchoconstrictor effects, CysLTs are also important modulators of vascular tone. The paradoxical effects of CysLTs in vascular preparations, namely contraction and relaxation, are now well documented in a number of vascular preparations from various species. Indeed, depending on the vascular tone, either under basal or norepinephrine (NE)-precontracted state, LTs are capable of inducing both vasodilatation and vasoconstriction (Smedegard et al., 1982). These effects vary among various species investigated (Feuerstein and Hallenbeck, 1987; Stanke-Labesque et al., 2000; Walch et al., 2000). Initially, it was viewed that vascular tissues acquire LTC_4 and

LTD_4 via intracellular transfer from leukocytes. Several studies have now demonstrated that CysLTs are generated in both EC and VSM tissues (Kuhn et al., 1983; Piper et al., 1983; Feuerstein, 1984; Piomelli et al., 1987). The main layer in the vascular wall, which produces LTs, is the adventitia, which is more abundant with tissue macrophages and mast cells. However, the medial layer is also capable of releasing 5-LO derived AA metabolites (Feuerstein, 1984).

1.2.6.3 Vasoconstrictor Responses to CysLTs- Role in Hypertension

The vascular preparations that showed profound vasconstriction to CysLTs were pulmonary veins and arteries (Hand et al., 1981; Hand et al., 1983), coronary vessels (Piomelli et al.,1987), cerebral arteries (Tagari et al., 1983), uterine arteries (Vincent et al., 1983), renal and mesenteric arteries (Rosenthal and Pace-Asciak, 1983; Schumacher et al., 1989). LTD_4 potentiates the responses to α-adrenergic agonists in rat aortic rings (Lawson et al., 1988). The presence of high affinity binding sites for [^{3}H]LTD_4 (linked to constrictor responses) have also been demonstrated in human saphenous vein and internal mammary artery (Allen et al., 1994). In some animal species, after systemic administration of CysLTs, there is an initial arteriolar constriction and rise in BP, followed by transient hypotensive period. This biphasic BP response to LTC_4 and LTD_4 is characteristic of guinea pig, monkey and sheep (Michelassi et al., 1982; Smedegard et al., 1982) but is uncommon in various strains of rats (Iacopino et al., 1983; Pfeffer et al., 1983). However, SHR are extremely sensitive to the cardiovascular effects of LTD_4. They exhibit a biphasic response. The initial pressor response was found to result from an

intense vasoconstriction in most peripheral vascular beds, which contributes to a rise in total peripheral resistance. The ultimate mechanism involved in the constrictor response to CysLTs in vascular tissues is not clearly established. The myotropic COX products, TXA_2 and PGs, mediate the major part of the constrictor actions of LTC_4 and LTD_4 in guinea-pig lung. In rabbit and rat lung, however, the CysLTs were much less potent in contracting parenchymal strips and there was little evidence of the release of COX products (Omini et al.,1981; Piper et al.,1981). LTD_4 evoked mesenteric vasoconstriction is also independent of thromboxane A_2 receptor activation (Schumacher et al., 1989). It has been shown that LTD_4 is a potent vasoconstrictor agonist in SHR strain; the mechanism of action seems to be mostly direct, but in part PG mediated (Zukowska-Grojec et al., 1982). The second hypotensive phase is associated with a progressive fall in cardiac output (CO) secondary to coronary vasoconstriction, reduced myocardial contractility and hemoconcentration, which may be caused by enhanced vascular permeability (Bayorh et al., 1984; Piper 1984; Zukowska-Grojec et al., 1985; Schumacher et al., 1989). LTD_4 evoked a much higher vasoconstrictor response in the perfused mesenteric vascular bed preparation isolated from 14 week old male SHR compared to the responses determined in preparations isolated from age-matched normotensive WKY strain (Shastri et al., 2001). While these observations are consistent with the presence of vascular CysLT receptors linked to vasoconstriction, their detailed characterization and likely alterations in SHR is presently unknown and the aim of the present thesis is to address this issue at the level of VSMC.

1.2.6.4 Vasodilator Effects of CysLTs

CysLTs are known to cause VSM relaxation in a variety of preparations from different species via two pathways which have currently been suggested to mediate this response. One involves the metabolites of AA via the COX enzymatic pathway and the other implicates products of L-arginine enzymatic pathway. Although both pathways may be present and active in the endothelium in different regional vascular beds, only one of these enzymatic pathways may be dominant and responsible for the relaxation observed (Walch et al., 2000). To characterize the influences of LTD_4 on regional vascular beds, the effects of LTD_4 on vasomotor tone in canine renal and superior mesenteric arterial rings were determined. In NE preconstricted rings, LTD_4 produced a dose-dependent relaxation in endothelium-intact ring preparations. Because relaxation induced by LTD_4 did not appear to be related to the release of COX metabolites (Secrest et al,1985). Later, they demonstrated that endothelium-dependent LTD_4-induced relaxation of canine superior mesenteric artery was linked to cyclic GMP production (Secrest et al., 1988). In pulmonary veins, the dominant pathway for CysLT relaxation was dependent on nitirc oxide (NO) generation (Ortiz et al., 1995). In addition to LTD_4, LTC_4 also showed endothelium-dependent relaxation in isolated thoracic aorta and pulmonary arteries of guinea pig. The vasodilator responses to these agonists were antangonized by LT antagonist, ICI-198615 (Sakuma, Gross et al., 1987; Sakuma and Levi, 1988). Together, these observations indicate the presence of CysLT receptors on the endothelium and that the responses may be mediated by NO generation (Walch et al., 2000). In contrast, both

LTD_4 and LTC_4 induced vasodilatation in isolated human saphenous veins were shown to be dependent on both NO and COX metabolite (Gleason et al., 1983). On the other hand, another report has suggested that LTC_4 induced relaxation is mediated by the release of metabolites of COX pathway in human saphenous veins (Allen et al., 1992), Thus CysLT may promote endothelium-dependent activation of these pathways and the extent of recruitment of each pathway may vary between different vascular beds and amongst various species. However, the receptors that mediate these divergent effects have not been characterized. In isolated human pulmonary artery and veins, $CysLT_2$-R activation has been shown to promote NO release. On the other hand, in guinea pig pulmonary artery and thoracic aorta, $CysLT_1$-R has been suggested to be responsible for the CysLT mediated relaxation (Walch et al., 2000)

1.2.6.5 Effects of CysLTs on Microcirculation

During systemic or local allergic reactions, LO products cause profound changes in capillary permeability, indicating the potential role of LO products in the modulation of microcirculation. They enhance the capillary permeability and promote plasma extravasation from the intravascular to exravascular compartment (Feuerstein, 1984; Feuerstein, 1986). Intradermal injection of LTs result in prolonged erythema, wheal formation and pain sensation, yet the wheal is characterized by pallor due to arteriolar constriction (Soter et al., 1983). Although the effect of LTs to enhance capillary permeability was observed in many species, including humans, some species seem to be less responsive (e.g., the rabbit) (Ueno et al., 1981). LTC_4, LTD_4 and LTE_4 are

extremely potent in increasing vascular permeability in rat and guinea pig. The microcirculatory effects of CysLTs may bear significance not only in inflammatory processes, but also in the promotion of edema, an important pathological factor in tissue injury. In addition, acute reduction in intravascular volume, loss of plasma proteins and impaired intracapillary oncotic pressure might well serve as additional factors in promoting CysLT-induced hypotension and shock (Feuerstein, 1984; Feuerstein, 1986).

1.2.6.6 Cardiac Effects of CysLTs

With the use of a variety of techniques, the presence of $CysLT_1$ and/or $CysLT_2$ transcripts in cardiac tissue has been identified (Heise et al., 2000; Kamohara et al., 2001; Lynch et al., 1999; Ogasawara et al., 2002). CysLTs have been shown to exert potent effects on the heart, contributing to heart failure (Kamohara et al., 2001). Leukotriene A_4 hydrolase plays a critical role in the generation of CysLT, and its expression is elevated in the heart of Ang II-induced hypertensive rats, suggesting that Ang II may promote cardiac CysLT production (Ishisaka et al., 1999). Moreover, low concentrations of CysLT, specifically LTD_4 and LTC_4, have been shown to promote a positive inotropic effect in the rat heart (Karmazyn and Moffat, 1990). CysLTs also regulate cardiac function by causing potent vasoconstriction of the coronary vascular bed both *in vivo* and *in vitro*. Similar to other actions of LTs, the strength of vasoconstriction varies in different species. Studies conducted in several *in vitro* preparations have clearly demonstrated that LTC_4, LTD_4 and LTE_4 are potent constrictors of the coronary arteries. They evoke a significant reduction in the coronary blood flow (Burke et al., 1982; Letts and Piper, 1982; Ezeamuzie and Assem, 1983; Roth and Lefer, 1983; Piomelli et al., 1987). A decrease in

contractility occurred with the reduction in flow, but there were no changes in heart rate (Feuerstein, 1984; Piper, 1984). Therefore, CysLTs are potent agents modulating coronary VSM tone; to the extent of causing profound inhibitory effect on myocardial function. However, some studies have also shown that CysLTs may also exert direct negative inotropic effect on the heart, which in turn may contribute to profound reduction in coronary blood flow (Burke et al. 1982). Isolated heart preparations from SHR show greater sensitivity to cardiodepressant action of LTD_4. Cardiac hypertrophy and coronary wall thickening have been reported in SHR strain which make them more prone to myocardial damage (Zukowska-Grojec et al., 1982). Because LTs are related in a variety of immunological and inflammatory reactions, their potent myocardial depressant effects may play a role in cardiac dysfunction associated with these reactions (Burke et al., 1982).

1.2.7 Relation between CysLT and Vasoactive Agonists

The pressor responses to sympathetic stimulation and exogenous agonists such as Ang II and AVP were remarkly inhibited in pithed SHR subjected to pretreatment with LTD_4 for 10 min (Bayorh et al., 1984). This was attributed to the effects of LTD_4 at various sites and these can be summarized as follows: i) generation of COX derived vasodilatory mediators (PGE_2 and PGI_2) in different vascular beds, ii) profound coronary vasoconstriction resulting in diminished CO, iii) increased permeability of the microcirculation resulting in plasma extravasation and tissue edema that could create mechanical interference to VSM contration, iv) attenuation of release of NE from peripheral sympathetic nerves (Bayorh et al., 1984). In contrast, a later study

demonstrated potentiation of the contractile effects of epinephrine and NE by LTD_4 in rat aortic rings and this potentiation was abolished by incubation with CysLT antagonist, FP-55712, but not by the COX inhibitor, indomethacin. These data suggested a specific cooperative interation of LTD_4 with α adrenergic agonist evoked responses possibly involving VSM CysLT-R activation by LTD_4 (Lawson et al., 1988).

Many studies have shown that LO derived metabolites could contribute to the vascular actions of Ang II (Stern et al., 1989). Recently, Stanke-Labesque et al first reported CysLT-dependent vasoconstrictor responses to Ang II in human internal mammary artery (Stanke-Labesque et al., 2000). Later, they showed that, although Ang II-evoked responses were similar between aortic rings of SHR and WKY, AA861 (5-LO blocker) or MK571 ($CysLT_1$ selective antagonist) abolished the responses to Ang II to an extent of 65% and 48% respectively in the SHR but not in WKY rings (Stanke-Labesque et al., 2001). Upon endothelium denudation, the inhibitory effect of MK571 was lost. Based on these data, they concluded that Ang II stimulates the release of a contractile factor from the endothelium (not ET-1) subsequent to CysLT generation and $CysLT_1$ activation (Stanke-Labesque et al., 2001). These studies were the first to support a direct link between Ang II – CysLT signaling and vasoconstriction in the blood vessels of SHR. Our laboratory has addressed this issue using a resistance type vascular bed preparation by examining this issue in perfused mesenteric vascular bed *ex vivo* after isolation from SHR and WKY strains (Shastri et al., 2001). The vasoconstrictor responses to Ang II, ET-1 in the MVB of SHR were significantly higher than WKY while the responses to KCl depolarization remained unaffected in the preparations of either strain. Addition of either

AA861 or MK571 abolished the exaggerated vasoconstrictor responses to Ang II but not to ET-1. In the same preparation, CysLT evoked vasoconstrictor responses were higher in the SHR with the rank order of efficacy being LTD_4> LTC_4> LTE_4. MK571 attenuated these responses. These data are consistent with the presence of vascular $CysLT_1$ receptor mediated exaggerated responses to Ang II in SHR. However, the underlining mechanism for the exaggerated response in SHR remains unknown.

Besides enhancing $[Ca^{2+}]_i$ level, studies have shown that Ang II elicits complex intracellular signaling events that include several mediators such as superoxide anions, cyclic nucleotides and alteration in NO levels (Touyz, 2000; Richard et al., 1998). In addition, stimulation of AT_1 receptors by Ang II leads to activation of several kinases, phospholipase (PLA_2) and phospholipase D (PLD), resulting in elevation of AA-derived metabolites such as CysLT (Freeman et al., 1998; Luchtefeld et al., 2003; Dulin et al., 1998; Lokuta et al.,1994; Nasjletti et al.,1998; Parmentier et al., 2001; Rao et al., 1994; Touyz 2000; Zafariet al.,1999). In addition to their well-known bronchiolar smooth muscle spasmogenic effect, CysLTs have been shown to enhance $[Ca^{2+}]_i$ levels and evoke vasoconstriction (Mazzetti et al., 2003; Bouchelouche et al., 2001; Kamohara et al., 2001; Ochsner et al.,1996; Ogasawara et al., 2002; Pedersen et al., 1997; Shastri et al., 2001; Stanke-Labesque et al., 2001). However, the evidence at the level of VSMC for the presence of CysLT receptors as well as regulation of CysLT production, CysLT receptor expression by Ang II have not been characterized so far. By monitoring the changes in $[Ca^{2+}]_i$ levels evoked by Ang II in both primary cultures of NRC and cultures

of ASMC, this study attempted to elucidate CysLT dependent responses to Ang II in these cells.

1.3 Roles of Ang II and CysLT in hypertensin

1.3.1 Roles of Ang II in SHR

SHR is an appropriate animal model of human essential hypertension. The pathogenesis of hypertension is multifactorial, as evidenced by studies showing polygenic inheritance (Dzau et al., 1992). The cellular and molecular mechanisms that contribute to hypertension still remain unknown. However, it is well accepted that increased peripheral resistance plays a critical role in blood pressure elevation. In SHR, the increase in vascular resistance has been attributed to multiple interacting factors, including structural alterations in small vessels, decreased endothelium-dependent vasodilation, and enhanced vascular reactivity to vasoconstrictor stimuli. (Folkow et al., 1982) Among the many vasoactive agonists that have been suggested in vascular hyperresponsiveness in hypertension, Ang II plays one of the most important roles. Whereas responses to ET-1, AVP, and NE have been demonstrated to be decreased, unchanged, or rarely increased, vascular reactivity to Ang II has been reported to be increased in vessels from SHR (Schiffrin et al., 1994; Touyz et al., 1996a; Resink et al., 1989). Altered Ang II–stimulated vascular responsiveness occurs early in the development of hypertension, and studies have shown increased $[Ca^{2+}]_i$ and contractile effects of Ang II in SHR as young as 6 weeks of age (Touyz et al., 1994; Chatziantoniouet al., 1990).

The signaling pathways responsible for enhanced Ang II–evoked excitation-contraction coupling in hypertension are not fully understood, and the signaling processes involved in the prehypertensive phase may differ from those in the established phase of hypertension. In cultured VSMC isolated from adult SHR, Ang II stimulated PLC–mediated signaling is increased, with enhancement of $[Ca^{2+}]_i$ and $[pH]_i$ responses relative to normotensive controls(Touyz et al., 1994; Heagerty et al., 1986). These effects are partially attributed to increased Ca^{2+} influx and mobilization and to enhanced activity of the Na^{+}-H^{+} exchanger (Rosskopf et al., 1993; Rembold, 1992), $[Ca^{2+}]_i$ elevation and alkalinization play very important roles in causing vasoconstriction (Touyz et al., 1994; Heagerty et al., 1986; Rosskopf et al., 1993; Rembold et al., 1992). They mediate actin-myosin interaction, crossbridge cycling, and VSM contraction (Rosskopf et al., 1993; Rembold et al., 1992) In addition to activation of the classic PLC–mediated signaling pathways commonly associated with Ang II, it has been evident that Ang II stimulates other signaling pathways including those dependent on tyrosine kinase and mitogen-activated protein kinase (MAPK). (Sauro et al., 1996; Berk et al., 1997; Butcher et al., 1993; Touyz et al., 1997a).

1.3.2 Role of Ang II Evoked AA-derived Metabolites in Hypertension

Besides PLC, the AT_1 receptor is also coupled to phospholipase (PLA_2) and phospholipase D (PLD) resulting in arachidonic acid (AA)-derived metabolites (Nasjletti et al., 1998; Touyz et al., 2000; Schlondorff et al., 1987; Rao et al, 1994). Ang II-evoked hypertrophic responses in rat VSMC are linked to elevations in non-cyclooxygenase

derived AA metabolites (Dulin et al., 1998; Muthalif et al., 1998; Zafari et al., 1999). There is evidence that Ang II-evokes free AA formation in both EC and VSMC and this in turn leads to the generation: i) cyclooxygenases derived metabolites: prostacyclin (PGI_2) and/or thromboxane A_2 (TXA_2) as well as other prostaglandins, ii) cytochrome P450 oxygenase derived metabolites that leads to generation of vasoconstrictor products: 20- and 19-HETEs, iii) region specific 5-,12-,15- lipoxygenases leading to the formation of -,12-,15-HPETEs and HETEs. While the vascular actions of PGI_2, TXA_2 as well as the role of 20-HETEs, have been well characterized, no studies have implicated a definitive role for 5-LO-derived leukotrienes, subsequent to the formation of 5-HETES, in Ang II-evoked responses in VSM tissues. A schematic diagram outlines how Ang II mediated generation of AA metabolites promotes CysLT generation (**Figure 1** page 18).

Non-selective lipoxygenase (LO) inhibitors such as phenidone reduced blood pressure (BP) in SHR and proteinuria in stroke-prone SHR (Nozawa et al., 1989; Munsiff et al., 1992). Ang II induced vascular hypertrophy and vasoconstriction were reduced by non-selective LO blockers (Natarajan et al., 1994; Stern et al., 1990; Takai et al., 1999). Enhanced pulmonary vascular reactivity to agonists and pulmonary hypertension in a rat model was attenuated by treatment with a 5-LO blocker (Voelkel et al., 1996). Recent studies by a group in France and work from our laboratory have shown that Ang II evoked vasoconstrictor responses in SHR were reduced by AA861, a selective 5-LO blocker (Shastri et al., 2001; Stanke-Labesque et al., 2001). Before describing these findings, a brief note on the pharmacology of CysLT actions will be presented.

1.4 Ang II action is CysLT dependent

1.4.1 CysLTs contribute to Ang II action in cardiovascular response

Understanding the pathophysiology of hypertension at the cellular and molecular level will help to reduce the burden of cardiovascular diseases. While the significant role of CysLT as key inflammatory mediators of bronchospasm has been well recognized, their contributions to the regulation of cardiovascular function has not been fully appreciated until recently (Shastri et al., 2001 & 2003; Liu et al., 2003; Stanke-Labesque et al., 2000 & 2001; Nasjletti**,** 1998; Natarajan et al., 1994; Stern et al., 1990; Allen et al., 1994). Recent work from our laboratory suggests that exaggerated Ang II evoked vasoconstrictor responses in the SHR are dependent on CysLT generation and activation of $CysLT_1$ receptors linked to Ca^{2+} mobilization. Among the well known Ca^{2+} mobilizing peptide agonists [Ang II, AVP and ET-1], Ang II plays a key role in the regulation of cardiovascular function. Most of the vascular actions of Ang II are mediated predominantly by the activation of the AT_1 subtype (Dzau, 1989; Oliver and Sciacca, 1984; Timmermans et al., 1993; de Gasparo et al., 1995). Consistent with this view is the demonstration by our laboratory that Ang II evoked much higher increases of $[Ca^{2+}]_i$ than AVP and ET-1 in VSMC and NRC in primary culture (Gopalakrishnan et al., 1991; Xu et al., 1991 & 1993). Most experimental studies focusing on the role of Ang II in hypertension utilize SHR as the primary model (Shastri et al., 2001; Stanke-Labesque et al., 2001; Timmermans et al., 1993; Touyz and Schiffrin, 2000). Ang II elicits complex, highly regulated intracellular signal transduction events that lead to both short-term (vasoconstriction and hemodynamic alterations) and long term biological effects

such as proliferation, hypertrophy, and migration as well as inflammation of VSMC. Touyz and Schiffrin have reviewed this subject (Touyz and Schiffrin, 2000). Besides activation of PLC to enhance $InsP_3$ and DAG production as primary second messengers, Ang II also activates a variety of kinases to regulate VSM growth, promotes apoptotic signaling in cardiac fibroblasts via expression of some proteins (Hao et al., 2000), and induces a hypoxic factor in VSMC (Richard et al., 2000). Ang II actions are also mediated by nitric oxide (NO) (Fernandez-Alfonso and Gonzalez, 1999), superoxide anions (Wang et al., 1999), cyclic nucleotides (Palaparti et al., 1999) and MAP kinases (Meloche et al., 2000). The major intent of the present thesis work is to examine whether CysLT would mediate Ang II-evoked responses in both cardiomyocytes and VSMC and whether this overactive pathway could contribute to exaggerated vasoconstrictor responses in the SHR.

1.4.2 CysLT contributes to Ang II evoked Ca^{2+} Signaling

Stimulation of AT_1 receptors by Ang II leads to activation of PLA_2 and PLD resulting in elevation of arachidonic acid (AA)-derived metabolites such as CysLT (Freeman et al., 1998; Luchtefeld et al., 2003; Dulin et al., 1998; Lokuta et al., 1994; Nasjletti et al., 1998; Parmentier et al., 2001; Rao et al., 1994; Zafari et al., 1999). CysLTs such as LTD_4, LTC_4 and LTE_4 exert their actions via activation of $CysLT_1$ and/or $CysLT_2$ receptors that are linked to PLC mediated Ca^{2+} mobilization (Heise et al., 2000; Lynch et al., 1999). CysLTs have been shown to enhance $[Ca^{2+}]_i$ levels and evoke vasoconstriction

(Mazzetti et al., 2003; Bouchelouche et al., 2001; Kamohara et al., 2001; Ochsner, 1996; Ogasawara et al., 2002; Pedersen et al., 1997; Shastri et al., 2001; Stanke-Labesque et al., 2001). In rat VSMC, the hypertrophic responses to Ang II is suggested to be at least partially linked to generation of non-COX derived AA metabolites (Dulin et al., 1998; Parmentier et al., 2001; Natarajan et al., 1994; Nozawa et al., 1990; Sasaki et al.,1997). Recent studies have demonstrated that Ang II-evoked vasoconstrictor responses in rat aortic rings and perfused rat mesenteric vascular bed were reduced in SHR strain by the inclusion of AA861, a selective inhibitor of 5-LO, or MK571, a selective $CysLT_1$ antagonist (Shastri et al., 2001; Stanke-Labesque et al., 2001). These data suggested that Ang II enhances the production of AA-derived 5-LO metabolites, CysLT, which may contribute to the exaggerated vasoconstrictor responses to Ang II in SHR (Shastri et al., 2001; Stanke-Labesque et al., 2001). Both Ang II and CysLT evoke intracellular Ca^{2+} mobiliztion. However, no study has so far demonstrated that Ang II evoked Ca^{2+} mobilization in rat cardiomyocytes is linked to CysLT mediated Ca^{2+} mobilization. In the present study, one of the goals was set to explore this posibility. The presence of 5-LO, $CysLT_1$ and $CysLT_2$ have been detected in VSMC especially in the diseased arteries (Luchtefeld et al., 2003; Allen et al., Circulation, 1998; Back et al., 2000; Walch et al., 2002; Mazzetti et al., 2003). However, whether Ang II evoked Ca^{2+} mobilization is linked to CysLT generation in ASMC remains unknown. Using minimally passaged cultured ASMC isolated from both WKY and SHR rats, we have attempted to address this issue.

2. PRESENT INVESTIGATION

2.1 RATIONALE FOR THE STUDY

Three major pieces of evidence provide the basis for detailed studies on Ang II - CysLT interactions in both NRC and ASMC.

1. CysLTs (LTD_4, LTC_4 and LTE_4) elicit vasoconstriction in both aorta and perfused mesenteric vascular bed. MK571 abolished the exaggerated responses to CysLT in the MVB of SHR confirming the presence of overactive vascular $CysLT_1$ mediated event (Shastri S et al, 2001). Therefore, detailed biochemical characterization of $CysLT_1$/$CysLT_2$ receptors in VSMC is important. Therefore, we initiated studies using minimally passaged cultured ASMC (between 2nd and the 3rd passages).

2. Interestingly, the exaggerated vasoconstrictor responses to Ang II but not ET-1 were reduced by either 5-LO blockade or $CysLT_1$ antagonism in the MVB of SHR. Whether $CysLT_2$ receptors are present and whether they contribute to, or oppose Ang II evoked vasoconstriction, has not been yet addressed.

3. Ang II evoked much larger increases in $[Ca^{2+}]_i$ than AVP and ET-1 in the primary cultures of NRC and ASMC (Xu et al., 1991 & 1993; Liu et al., 1999). The hypertrophic response evoked by Ang II in NRC and ASMC are attributed to elevated $[Ca^{2+}]_i$ evoked by Ang II (Sadoshima et al., 1995 a & b). Low concentrations of LTD_4 and LTC_4 have been shown to promote a positive inotropic effect in the rat heart (Karmazyn et al., 1990).

Despite this evidence, there are no studies to demonstrate whether Ang II evoked Ca^{2+} mobilization is linked to CysLT generation in NRC and ASMC. While increased cardiac LTA_4 hydrolase activity has been detected in rats that were made hypertensive by Ang II infusion (Ishizaka et al., 1999), whether NRC and ASMC express CysLT receptors and whether Ang II evoked $[Ca^{2+}]_i$ responses would be mediated by CysLT has not been addressed. Therefore, the major aim of the present work is to address these key issues at the levels of NRC and VSMC.

Based on the above, the following working hypotheses have been formulated.

2.2 WORKING HYPOTHESES

1. CysLTs are generated in both ASMC and NRC. Both $CysLT_1/CysLT_2$ receptors are present on ASMC and NRC. Activation of these receptors result in increases in $[Ca^{2+}]_i$ levels. There is increased number of cell surface receptors and/or increased expression of CysLT receptors in the ASMC of adult SHR in established phase of hypertension compared to age and sex matched normotensive WKY.

2. AT_1 receptor mediated increases in $[Ca^{2+}]_i$ response evoked by Ang II is linked to AA-derived, 5-LO-dependent, CysLT generation in both NRC and ASMC. CysLT dependent $[Ca^{2+}]_i$ response to Ang II is exaggerated in cultured ASMC derived from SHR. Inclusion of either AA861 (5-LO inhibitor) or the 5-LO activating protein (FLAP) inhibitor, (MK591), or the $CysLT_1$ selective antagonist, MK571, attenuates this indirect CysLT mediated exaggerated peak $[Ca^{2+}]_i$ responses to Ang II in both NRC and ASMC. The blockade of peak $[Ca^{2+}]_i$ responses to Ang II is more pronounced in the ASMC of SHR compared to the responses seen in ASMC isolated from normotensive WKY.

2.3 BRIEF EXPERIMENTAL STRATEGY/DESIGN

Our laboratory is adequately equipped for monitoring the basal and agonist(s)-evoked changes in cytosolic free calcium, $[Ca^{2+}]_i$, levels in the primary cultures of NRC and minimally passaged cultured ASMC (Liu et al, 1999). Therefore, the major goal of the present study is to examine whether LTD_4 and LTC_4 evoke increases in $[Ca^{2+}]_i$ in NRC and ASMC in addition to examining whether Ang II evoked peak $[Ca^{2+}]_i$ responses would be attenuated by AA861, MK591, MK571 and Bay u9773. This is the core data and premise for expanding the scope of this project and the rationale for a more detailed study at the level of expression of CysLT receptors and its regulation. The following experiments constitute the strategy for the work.

1 Preparation of primary cultures of NRC and measurement of $[Ca^{2+}]_i$ changes to agonists (AVP, Ang II and ET-1) in both multiple and single cells using fura-2 fluoresence measurement.

2 Isolation and culture of ASMC from SHR and WKY strains and measurement of $[Ca^{2+}]_i$ signals to agonists (AVP, Ang II and ET-1) in both multiple and single cells.

3 Measurement of basal and agonist(s)-evoked changes in CysLT production in the culture medium of NRC and ASMC of SHR and WKY strains and whether it is regulated by AA861 and MK591. CysLT levels are monitored using an enzyme immuno assay kit.

4 Levels of expression of $CysLT_1$ and $CysLT_2$ in NRC and ASMC will be examined.

In addition, the differences in the levels of $CysLT_1$ and $CysLT_2$ mRNA expression between ASMC of SHR and WKY strains will be evaluated. cDNA probes for human $CysLT_1$ and $CysLT_2$ have been obtained as a gift from Dr. Garry O'Neill, Merck-Frosst Canada and the probes have been amplified. $CysLT_1$ and $CysLT_2$ mRNA levels will be determined using Northern blot analyses.

3. MATERIALS AND METHODS

3.1 Animals

The care and use of animals conformed to the regulation stipulated by the University of Saskatchewan Animal Care Committee, which is similar to the guidelines established by the US National Institute of Health for the maintenance and use of experimental animals in our laboratories (NIH Publication No. 85: 23, 1996). Sprague-Dawley rats (both male and female), Male SHR and WKY rats were obtained from Charles River (St. Constant, Quebec, Canada) or from Harlan (Indianapolis, Indiana, U.S.A.) at an age of about 10 to 12 weeks. They were housed in our animal center assigned to the Department of Pharmacology with due care under standardized conditions with a light/dark cycle of 12 h and a constant temperature of 22 ± 1 °C. The rats were fed ad libitum with food pellets (Purina Rat Chow) and tap water. The Sprague-Dawley rats (12 to 16 weeks) were used for breeding the neonatal rats. The SHR and WKY rats were utilized for experiments when they reached the age between 14 and 16 weeks.

3.2 Chemicals and Reagents

3.2.1 Agonists

Angiotensin II (Ang II), arginine vasopressin (AVP), and endothelin-1 (ET-1) were from Bachem (Torrance, CA). LTC_4, LTD_4 and LTE_4 were obtained from Cayman Chemicals, Ann Arbor, MI, USA.

3.2.2 CysLT synthesis inhibitors and receptor antagonists:

The 5-LO inhibitor, AA-861 (2-[12-hydroxydodeca-5,10-diynyl]-3,5,6-trimethyl-p-benzoquinone) was obtained from Cayman Chemicals, Ann Arbor, MI, USA. Propionic acid,3-[[[3-[2-(7-chloro-2-quinlinyl)ethinyl]phenyl][[3-(dimethylamino)-3-oxopropyl] thio] methyl] thio]-,(E)- sodium salt (MK571 or otherwise called L660711), the FLAP inhibitor,(3-[1-(4-chlorobenzyl)-3-(t-butylthio)-5-(quinolin-2-yl-methoxy)-indol-2-yl]2,2-dimethyl propanoic acid) (MK591) and losartan (AT_1 selective antagonist) were received as gifts by the kind courtesy of Merck-Frosst, Pointe Claire, Dorval, Quebec, Canada. BAY u9773 was obtained from Biomol Reseaerch Laboratories (Plymouth Meeting, PA). PD123319, 2-aminoethoxydiphenyl borate (2-APB) were purchased from Sigma Chemical Co (Oakville, ON., Canada).

3.2.3 Reagents for cell culture

Bromodeoxyuridine, collagenase (Type II), elastase (Type IV), soybean trypsin inhibitor, were purchased from Sigma Chemical Co (Oakville, ON., Canada). Trypsin, culture media, DMEM, new born calf serum, horse serum, penicillin-streptomycin-fungizone and glutamine were from GIBCO-BRL (Life-Technologies, Grand Island, New York, U.S.A.).

3.2.4 Kits and reagents for northern blotting analysis

RNA STAT-60^{TM} for total RNA extaction was the product of Tel-Test, Inc. (Friendswood, TX). Northern Max^{TM} kit, Brightstar^{TM}-Plus positive charged nylon

membranes, DECAprime TM II kit, and NucAway^{TM} Spin Column were products of Ambion. [$\propto$-^{32}P] dCTP (3,000 Ci/mmol, 10 μCi/μl) was purchased from Amersham Pharmacia Biotech , Inc. (Baie d'Urfe, Quebec, Canada). QIAprep Spin Miniprep kit and QIAEX II Gel Extraction kit were purchased from QIAGEN, Inc. (Mississauga, Ontario, Canada).

3.2.5 Other chemicals and kits

Fura-2 AM and pluronic acid F-127 were from Molecular Probes (Eugene, OR). Analytical grade salts for the preparation of Krebs buffer were purchased from Sigma Chemical Co (Oakville, ON., Canada), BDH (Dartmouth, NS, Canada), Calbiochem (La Jolla, CA), BRL (Burlington, ON) or ICN Biomedicals Inc. (Costa Mesa, CA). The EIA kit for the measurement of total CysLT levels were obtained from Cayman Chemicals, Ann Arbor, MI, USA.

3.3 Methods

3.3.1 Cardiomyocyte culture

The details of isolation and primary culture of ventricular myocytes from newborn (3 days old) Sprague-Dawley rats have been described earlier (Liu et al., 1999; Xu YJ et al., 1991 & 1993). In the present study, we improved the isolation procedures to prepare either purified cardiomyocytes or cardiac mesenchymal cells by introducing the modifications adopted by others (Lau et al, 1980; Simpson and Savion, 1982). Thirty to fourty newborn 1-3 day old Spragur-Dawley rats were anesthetized and cardiac ventricles were removed

and placed in a petri dish containing 10 ml heparinized phosphate-buffered saline under aseptic conditions followed by washing with the buffer for 3 times. The tissues were cut to 1 mm^3 pieces and transferred to 25 ml of 0.10% trypsin solution. The tissues were digested for 10 min at 37°C with stirring, the first digestion solution was discarded and the subsequent supernatants were collected at 10 min time intervals. After centrifugation at 600 x g for 3 min., the cell pellet was resuspended in DMEM and incubated in two to three 75 cm^2 flasks for 3 hours at 37°C. While the mesenchymal cells attached to the flasks first, myocytes did not adhere during this short duration of incubation. The attached mesenchymal cells were cultured in DMEM with 10% fetal calf serum. These cells were utilized in a few selected experiments to characterize $[Ca^{2+}]_i$ response to agonists such as Ang II, AVP and ET-1. The unattached cardiomyocyte cell suspension was layered on either 6 well culture plates for CysLT assay or on flaskes or glass coverslips for fluorescence measurement. Therefore, cells grown by this by this method contained 75-80% cardiomyocyte by day 5 in primary culture. When bromodeoxyuridine (0.1 mM) was included, it selectivively inhibited the proliferation of a few mesenchymal cells and thus led to the presence of purified myocyte-rich (95% purity) preparations relatively devoid of fibroblasts or mesenchymal cells (Liu et al, 1999).

3.3.2 ASMC isolation and culture

The details of isolation and culture of ASMC from WKY and SHR rats have been described (Gopalakrishnan et al., 1991). Rats were exposed to anhydrous diethyl ether until the induction of anesthesia and were immediately killed. All subsequent procedures

were done under sterile conditions inside the flowhood. Aortic vessels were carefully dissected out and cleared of fat. After the intimal layer was gently scraped, the vessels were cut longitudinally and incubated for 10 min at 37^{O}C in Hanks' balanced salt solution (HBSS) containing 0.2% type II collagenase, 0.025% type IV elastase, and 0.02% soybean trypsin inhibitor (all from Sigma Chemical). This ensured removal of the endothelial cells and the vessels were cleaned further to remove the remaining adventitia and fat using the teflon scraper. The cleaned vessels were cut into small pieces (1-2 mm in length), digested in the fresh HBSS enzyme solution for 45 to 60 min at 37^{O}C. The suspension was centrifuged at 200 x g for 5 min, and the pellet was resuspended in 10 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) new born calf serum and 10% (vol/vol) horse serum, L-glutamine (2mM), penicillin (100U/ml), streptomycin (100 µg/ml), and Fungizone (1µg/ml). The dispersed cells were plated into a tissue culture flask and incubated at 37^{O}C in a humidified 5% CO_2 / 95% O_2 atmosphere. After the cells adhered sufficiently to the bottom of the flask (3-4 days), the remaining cell and tissue debris were removed and replaced with fresh culture medium.

3.3.3 ASMC passage and maintenance

Confluent monolayers formed between 6 and 8 days after isolation. This was referred to as the primary culture. When the cells reached the stage of confluence, the culture medium was removed and the cells were treated with 0.06% trypsin for 2-3 min at 37^{O}C until the cells started to show signs of detaching. This stage was identified morphologically under the microscope as and when the spindle shaped attached cells became rounded. Trypsin was then quickly removed and the cells washed once with

DMEM. Fresh culture medium was added to the flask and the cells were detached by scraping with a teflon scraper and resuspended by trituration using a transfer pipette. The cells were then replated (in culture medium) at a split ratio of between 1:3 and 1:6 in either 6 well plates or 75 cm^2 culture flasks. The culture medium was changed every 2-3 days (throughout the period of maintenance of ASMC) and the cells multiplied to reach confluency in 3-5 days for each passage level. Sub-cultured cells were used between the 2^{nd} and 3^{rd} passage levels. We have previously ascertained that $[Ca^{2+}]_i$ responses to agonists were not significantly different between cells in primary culture and those maintained up to the fifth passage. Smooth muscle morphology was routinely assessed by examination under microscope, and the level of contamination by fibroblasts was <3% of the total population of cells. Moreover, occasional verification studies confirmed that >90% of cells showed positive immunoflourescent staining with α-actin antibody, confirming smooth muscle characteristics of adherent cells.

3.3.4 Cell viability examination

Cell viability was routinely checked prior to experiments by trpan blue exclusion criteria. Dead or dying cells took up the blue dye while viable cells remained intact. Cell number was counted using a hemocytometer, Fisher counter and a microscope.

3.3.5 Determination of $[Ca^{2+}]_i$ using Fura-2 in dispersed NRC or ASMC

The NRC or ASMC which were grown on flasks and reached confluence were maintained in serum-free DMEM for 24 hr. The culture medium was removed and the cells were treated with trypsin at 37°C for 3 min. The cells were transferred to Krebs HEPES buffer

(composition in mM: NaCl 145, KCl 5, $CaCl_2.2H_2O$ 1.8, $MgCl_2.6H_2O$ 1.2, glucose 10, HEPES 10, and 0.2% bovine serum albumin [BSA] - pH 7.4), centrifuged (600 x g for 3 min at 4°C), and resuspended in the same buffer at a cell density of ~1.0^6 cells/ml. Fura-2 acetoxy methyl ester (Fura-2 AM, Molecular Probes) prepared in dimethyl sufoxide (DMSO) at a working stock concentration of 5 mM was added. To facilitate the solubility of Fura-2 AM and ensure effective loading, a small concentration of the surface agent, pluronic F 127 (0.02%), was added to the fura-2AM solution. The final concentration of Fura-2 AM was 5 μM in the incubation at 37°C for 30 min. The cells were washed twice, centrifuged, and resuspended in the same buffer at a cell density of ≈ 1.0^6 cells/ml. The excitation signals (340/380nm) were determined using a fluorimeter designed to monitor fura-2 fluorescence (JASCO CAF-100 Ca^{2+} Analyzer, Japan Spectroscopic, Tokyo, Japan). Details of calibration and determination of basal and agonist(s)-evoked increases in $[Ca^{2+}]_i$ levels have been described earlier (Liu et al, 1999; Xu et al, 1991) as well as listed as below.

3.3.6 Determination of $[Ca^{2+}]_i$ using fura-2 fluoresence in adherent NRC

After NRC grown on glass coverslips attained confluence (3 days), the cells were maintained in serum free medium for 24 hr. The cells were washed twice in Krebs-HEPES buffer (composition in mM: NaCl 145, KCl 5, $CaCl_2.2H_2O$ 1.8, $MgCl_2.6H_2O$ 1.2, glucose 10, HEPES 10, and 0.2% bovine serum albumin [BSA] - pH 7.4). Cells were loaded in the dark with fura-2 acetoxymethyl ester (final 5 μM) for 30 min followed by three buffer washes. Coverslips were inserted into a microcuvette containing 500 μl

buffer at 37^{O}C. The excitation signals (340/380nm) were determined using the fluorimeter described above. Details of calibration and determination of basal and agonist(s)-evoked increases in $[Ca^{2+}]_i$ levels have been described earlier (Liu et al, 1999; Xu et al, 1991) as well as listed as below.

3.3.7 Fura-2 Fluorescence Measurement

Experiments with dispersed cells were performed with a fluorimeter specially designed to monitor fluorescence (JASCO CAF-100 Ca^{2+} Analyzer, Japan Spectroscopic, Tokyo, Japan). The detailed methodology regarding the use of this equipment has been described earlier (Gopalakrishnan et al., 1991; Xu et al, 1991; Batra et al., 1993). For each determination, 400 µl of Fura-2 loaded NRC or ASMC cell suspension (containing 0.2 – 0.3 X 10^6 cells) was added to a cuvette maintained at 25°C with a stirring rate at 800 rpm. Our previous study have confirmed that no significant differences in Fura-2 fluorescence were noticed at either 25°C or 37°C, and dye leakage and photobleaching were minimal at 25°C. Therefore, we carried out all the experiments at 25 °C. The cells were subjected to excitation at 340 nm and 380 nm, and the emitted light collected at the photomultiplier through a 500 filter. Free fura-2 maximally excites at 380 nm, whereas the Ca^{2+} bound form is maximally excited at 340 nm. Thus, the 340/380 fluorescence ratio is a measure of fura-2 chelation to Ca^{2+} which is directly proportional to the rise in the levels of free Ca^{2+}. The ratio of fluorescence due to excitation at 340 nm to that at 380 nm ($R_{340/380}$) was calculated by the analyzer. Since intracellular free fura-2 is essentially trapped in the

cytosolic compartment and available to chelate Ca^{2+} only in this compartment, free Ca^{2+} can therefore be considered cytosolic free Ca^{2+}, or $[Ca^{2+}]_i$. The basal level and the peak increase in the ratio of fluorescence were noted from the display monitor for the calculation of basal and maximal increases in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ levels at basal and at the maximal increase evoked by agonists were calculated according to the formula $[Ca^{2+}]_i = K_D \times (R-R_{min})/ (R_{max} - R) \times Sf_2/Sb_2$ (Grynkiewicz et al., 1985). R_{max} was obtained after the addition of 25 µl Triton-X-100 (10%), and R_{min} was determined by adding 25µl of 100 mM EGTA (ajusted to pH8.5 with Tris Base). K_D (224 nM) is the affinity for fura-2 binding to Ca^{2+}, R represents the measured cellular ratio of fluorescence (340/380) or relative fluorescence detected in mode II. R_{max}, R_{min} are ratios obtained in Ca^{2+} saturating conditions in the presence of Triton X-100, and Ca^{2+} free conditions in the presence of EGTA respectively. Sf_2 and Sb_2 values are the 380 nm excitation signals in the absence and presence respectively of saturating concentrations of Ca^{2+} determined in mode III.

3.3.8 Single Cell Fura-2 Imaging

NRC or ASMC grown on glass coverslips (Delta T Dish 0.15nm, Bioptechs Inc., Butler, PA) were maintained in DMEM for 3 days and changed to serum free medium for 24 hr. The cells were washed in Krebs-HEPES buffer and subjected to fura-2 loading and washing. The dish was mounted on the stage of an inverted Olympus I X 70

epifluorescence microscope fitted with a UA po 20 x .5 objective. The fura-2 fluorescence images were acquired using a fast monochromatic integral 125W Xenon light source (SpectraMaster Monochromator, Life Science Resources, Perkin Elmer, Gaithersburg, MD) with a shutter speed for monitoring alternate 340 and 380 nm excitation signals every 200 msec. Ratiometric signals at 340/380nm were acquired at a rate of 3 images per second. The emission signal at 510nm was collected using a charge-coupled device camera (Astrocam, Cambridge, England, UK). The digitized signals were stored and processed using Ultra*VIEW*^{TM} Imaging System software (Wallac Imaging, Perkin Elmer Life Sciences, Gaithersburg, MD). Ang II (50 nM) was added to the coverslips after acquiring images for the first 30 seconds to determine basal fura-2 fluorescence. Interacting agents were added to fura-2 loaded cells for 3 min prior to imaging. R_{max} and R_{min} values were determined using Ca^{2+} ionophore, bromoA23187 (50 μM), and then by quenching with 50 μl of Tris (50 mM), EGTA (100 mM) solution (pH 8.5) at the end of each experiment. Once these values were entered, the software program employed provided the absolute $[Ca^{2+}]_i$ values for each cell using the Grynckiewicz equation.

3.3. 9 Determination of Concentration-Response (C-R) Curves

The concentration-response (peak $[Ca^{2+}]_i$) curves to agonists (Ang II, AVP, ET-1, LTD_4 and LTC_4) were determined by successive incubations under similar conditions. CR determinations for LTC_4 and LTD_4 were performed in the buffer medium devoid of BSA. The same aliquot of cell suspension loaded with the same concentration of Fura-2 was

employed, thus it is reasonable to compare the results of graded C-R curves. Basal fluorescence ratio prior to agonist inclusion followed by the changes in fluorescence ratio after the addition of agonist, was recorded for a period of 4 to 5 min. The maximal increase in flurescence ratio (peak ratio) evoked by the agonist was noted, and these ratios were converted to levels according to Grynkiewicz et al., (1985). The CR determinations to agonists were also performed in the presence of optimal concentration(s) of 5-LO inhibitor, AA861 (either 10 or 30 μM) (Shastri et al., 2001; Stanke-Labesque et al., 2001; Walker et al., 2002), $CysLT_1$ antagonist, MK571 (100 nM) (Lynch et al., 1999; Ruck et al., 2001; Shastri et al., 2001; Stanke-Labesque et al., 2001), dual $CysLT_1$/$CysLT_2$ antagonist, BAY u9773 (100 nM) (Heise et al., 2000), AT_1 selective antagonist, losartan (1 μM;) (Booz et al., 1996; Kem et al., 1991; Sadoshima et al., 1993a & 1995b), AT_2 selective antagonist, PD123319 (1 μM) (Booz et al., 1996; Kem et al., 1991;Goldenberg et al., 2001; Sadoshima et al., 1993a & 1995b; Touyz et al., 1996b), and the cell permeating inositoltrisphosphate ($InsP_3$) blocker, 2-aminoethoxydiphenyl borate (2-APB, 50 μM) (Missiaen et al., 2001). The concentrations of all these agents were carefully chosen to ensure their selectivity of inhibition/blockade as validated by previous reports. Each agent was maintained in the cuvette for 3 min prior to agonist challenge. In select experiments, the fura-2 loaded cells were washed and placed in Ca^{2+} free buffer with 1 mM EGTA (pH 7.4) in the cuvette for 15 min prior to agonist challenge and fluorescence measurement. All the inhibitors or antagonists were added to the cell suspension 3 min. prior to the addition of the agonists and the corresponding fluorescence ratios were monitored.

3.3.10 Total CysLT measurement

NRC or ASMC grown on 6-well culture plates for 3 days (~ 0.3 - 0.5 x 10^6 cells/well) were maintained for the last 24 hr in 2 ml medium devoid of serum. The medium was replaced with Krebs-HEPES buffer for the last 2 hr prior to stimulation with Ang II (100 nM) for varying time intervals (from 5 to 360 seconds). CR determinations for Ang II (100 pM -1 μM), AVP (100 pM – 1 μM) and ET-1 (100 pM – 1 μM) evoked increases in CysLT levels were determined 1 min after the addition of respective agonist(s) and all these assays were performed in duplicate. The responses to Ang II were also determined in the presence of AA861 (10 μM), MK591 (100 nM), losartan (1 μM) or PD123319 (1 μM). A 450 μl aliquot of culture medium was stored in siliconized tubes at –80^{o}C. Total CysLT levels were determined within ten days of storage by enzyme immunoassay (EIA) following the protocol provided by Cayman Chemicals (Ann Arbor, MI). Total CysLT levels were assayed spectrophotometrically (405 nm for measurement of acetylcholinesterase activity) as outlined in the kit using Anthos HT1 96 well microplate reader (Anthos Labtec Instruments, Salzburg, Austria). The lowest detection limit was 4.0 pg/ml and the 50% (B/B_0) ratio was 40 pg/ml. The intra and inter assay coefficients of variation were 7.3 ± 2.5% and 5.8 ± 2.9% respectively. Cells were counted and the data normalized to express the values as CysLT/ml /million cells.

3.3.11 Northern Blot $CysLT_1$ / $CysLT_2$ mRNA Measurement

3.3.11.1 Total RNA extraction

Total RNAs of NRC and ASMC were extracted as previously described (Chomzynski & Sacchi, 1987) by using RNA STAT 60^{TM} reagent. The instructions for the extaction were provided in the manual. Serum-free DMEM was removed from the culture flasks and cells were lysed directly in flasks by adding the RNA STAT-60^{TM} (3 ml/75 cm^2 flask) and passing the cell lysate several times through a pipette. The washing of cells before addition of the RNA STAT-60^{TM} should be avoided as this increases the possibility of mRNA degradation. The cell lysate was transferred to a sterilized 14 ml centrifuge tube and stored for 5 min. at room temperature to permit the complete dissociation of nucleoprotein complexes followed by addition of 0.2 ml of chloroform per 1 ml of the RNA STAT-60^{TM}. The sample was covered tightly and shaken vigorously for 15 seconds and allowed to stay at room temperature for 2-3 min. The samples were centrifuged at 12,000 g (maximum) for 15 min at 4°C. Following centrifugation, the homogenate separates into two phases: a lower red phenol chloroform phase and the colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interphase and organic phase. The volume of the aqueous phase is about 60% of the volume of RNA STAT-60^{TM} used for lysing the cells. The aqueous phase was trasnsferred to a fresh sterilized tube and mixed with isopropanol (0.5 ml of isopropanol per 1 ml of the RNA STAT-60^{TM} used). The samples were left at room temprature for 5-10 min and then centrifuged at 12,000g for 10 min. at 4°C. RNA

precipitate (often visible before centrifugation) forms a white pellet at the bottom of the tube. The supernatant was removed and the RNA pellet was washed once with 75% ethanol and the supernatant was discarded and the pellet was resuspended, vortexed and subjected to subsequent centrigugation at 75,00 x g for 5 min at 4 °C. Then, 1 ml of 75% ethanol per 1 ml of RNA STAT-60^{TM} was added and it was subjected to homogenization. At the end of procedure, the RNA pellet was briefly subjected to air-drying and dissolved in diethylpyrocarbonate (DEPC) treated Rnase-free water.

3.3.11.2 Quantitation of Isolated total RNA

The amount and purity of total RNA was determined by spectrophotometer. The concentration of RNA in solution was calculated based on an optical density of 1.0 being equal to 40 µg/ml RNA. The purity was assessed as the ratio of fluorescence at 260 nm to that at 280 nm.

3.3.11.3 RNA electrophoresis

Isolated total RNA was electrophoresed using the protocol described ealier (Pelle and Murphy, 1993). RNA samples (50 µg/well) and molecular weight standards were first mixed with 3 times the volume of formaldehyde loading buffer provided with the NorthernMax^{TM} kit. To denature the RNA secondary structure, the mixture was incubated at 65°C for 15 min followed by rapid transfer to ice. The sample was then loaded onto 1.0% agarose gel dissolved in denaturing gel buffer with 0.1 µg/ml ethidium bromide. MOPS gel running buffer was employed for the electrophoresis. The

electrophoresis was carried out at a charge of 5V/cm of gel allowing the RNA to traverse the gel. The gel was then visualized under *uv* light and photographed.

3.3.11.4 Transfer of RNA to nylon membrane

The RNA was transferred from the agarose gel to an Ambion's BrightStar-Plus™ membrane by using a downward transfer assembly modified from the method develpoed by Chomczynski (1992) and following the procedures provided with the kit. After transfer, the RNA on the membrane was crosslinked using a U.V. Stratalinker (1,200 joules).

3.3.11.5 Preparation of probes

3.3.11.5.1 Bacterial trasformation

100 ng of plasmid DNA containing either human $CysLT_1$ or $CysLT_2$ cDNA fragments was added to 200 µl of DH5α competent cells mixed and stored on ice for 30 min. The mixture was then incubated at 42°C for 90 seconds and then quickly chilled on ice for 1-2 min. 1 ml of LB medium (1% bacto-trpptone, 0.5% bactoyeast extract, 1% NaCl, pH 7.0) was added to each mixture. The competent cells were then incubated at 37°C with shaking at a rate of 225 rpm for 1 h. Different volume of transformed bacterial cells were spread over the surface of agar LB medium on 1.5 % agar with 100µg/ml ampicillin. The plates were incubated overnight at 37°C in an inverse position.

3.3.11.5.2 Isolation of plasmid DNA

The procedure for isolation of plasmid DNA was modified from an earlier methodology (Sambrook et al., 1989). Different colonies picked up from each plate were cultured overnight at 37°C with vigorous shaking in 10 ml LB medium with 100µg/ml ampicillin. 5 ml of the culture was centrifuged at 12000g at 4°C for 10 min. The plasma DNA was isolated by using a QIAprep Spin Miniprep kit and following the protocol provided with the kit. The isolated DNA was dissovled in 50µl elution buffer (10 mM Tris-.Cl, pH 8.5) and stored at -20°C.

3.3.11.5.3 Recovery of $CysLT_1$ or $CysLT_2$ cDNA fragments from plasma DNA

Both $CysLT_1$ and $CysLT_2$ cDNA were recovered from the corresponding plasmid DNA by a digestion at 37 °C for 1 h using restriction enzyme Pme I. The cDNA were separated by 1% agrose gel electrophresis. Bands of cDNA fragments of $CysLT_1$ and $CysLT_2$ receptor were cut under U.V. light. The cDNA fragments were then isolated and purified from the gel by utilizing a QIAEX II Gel Extraction kit by following the procedures provided with the kit. The purified cDNA fragments were finally dissolved in 40 µl elution buffer (10 mM Tris.Cl, pH 8.5) and the concentrations were determined by spectrophotometer following the procedure described earlier. An optical density of 1.0 corresponds to 50 µg/ml double stranded DNA. The cDNA was then stored at -20°C.

3.3.11.5.4 Radiolabeling of $CysLT_1$ or $CysLT_2$ cDNA

Labeled nucleotides were incorporated in DNA synthesized by an oligo-labeling method (Feinberg & Vogelstein, 1983). The DECAprimeTM II random priming DNA labeling kit (Ambion) was used. 25 ng of cDNA was used for labeling. The cDNA fragment solution was mixed with 2.5 µl of 10X decamer solution heated in at 95°C for 3 min and then chilled on ice. 10 µl of denatured DNA/Decamer Mix, 5.0 µl of 5X Reaction buffer containing unlabeled dNTP's mixture, 5.0µl [$\propto$-^{32}P]dCTP (3,000 Ci/mmol, 10 µCi/µl), 1.0 µl Exonuclease-free klenow fragment of DNA Polymerase I and 4.0µl of nuclease-free H_2O were then mixed and followed by incubation for 10 min at 37 °C. The reaction was terminated by adding 1 µl of 0.5 M EDTA. The radiolabeled probe was then separated from the incorporated dCTP through a NucAway^{TM} Spin Column.

3.3.11.6 Northern hybridization

Northern blotting was performed using Northern Max^{TM} kit following the instructions provided with the kit. A sample of 60 µg total RNA was denatured and size-fractionated on a 1% formaldehyde-agarose gel, transferred onto the Brightstar-Plus^{TM} positive charged nylon membranes and immobilized by UV crosslink. $CysLT_1$, $CysLT_2$ and β-actin DNA probes were labeled with ^{32}P using DECAprime TM II kit. A minimum of 10 ml of ULTRAhyb solution per 100 cm^2 of membrane was used and the membrane was prehybridized for 30 min at 42°C. The labeled probes were diluted 10 times with 10 mM EDTA and incubated at 90 °C for 10 min followed by addition of 1.0 ml of ULTRAhyb. The denatured probe was transferred to the hybridization solution. The blot was hybridized overnight (20 hr) at 42°C. The membrane was washed twice for 5 min at

room temperature using the Low stringency Wash Solution which is equivalent to 2 X SSC, 0.1% SDS. Then the membrane was washed twice for 15 min at 42°C with High Stringency Wash Solution which is equivalent to 0.1 X SSC, 0.1% SDS. The membranes were exposed to X-film at –70 °C in the presence of an intensifying screen for 24 hrs followed by visualization and quantification using a Phosphor Imager (Molecular Dynamics).

3.4 Statistical analysis

Experimental values are reported as means ± SEM of a minimum of five separate experiments performed on different days using different batches of NRC and ASMC. Comparison of mean values was performed by ANOVA (Super ANOVA software). Simultaneous multiple comparisons were assessed using Scheffe's F-test and the concentration of agonist required to produce 50% of the maximal response (EC_{50}) and the maximal increase in $[Ca^{2+}]_i$ (E_{max}) values attained for each agonist were derived from log CR curves.

4. RESULTS

4.1 Results from Studies Using NRC

4.1.1 Comparison of the characteristics of myocytes and non-myocytes (fibroblasts)

The distinguishing characteristics of either purified myocytes or non-myocytes maintained in primary culture are shown (Table 1 and Figure 2). Inclusion of 0.1 mM BrdU led to single or clusters of myocytes in culture that showed distinct beating characteristics with cells depictiting the morphological features of a round small nucleus with either one or at the most two nucleoli. In addition, the cytoplasm stained red with myocytes under Mayer's haemalum-eosin staining. These features are consistent with the cells having characteristics of myocytes as shown in pictures (Figure 2a and c) under high and low powers respectively. In contrast to myocyte morphology, the non-myocytes showed characteristics of oval larger nucleus with two or more nucleoli (as shown in Figure 2b). In addition, the cytoplasm stained pale blue in the presence of Mayer's haemalum-eosin staining. Thus, in the absence of 0.1 mM BrdU in the medium, non-myocyte cells proliferated such that the myocyte preparations yielded population of cells with 10-20% contamination of non-myocytes (Figure 2d). Thus, purified myocytes were utilized in most of the experiments described in the present study. Studies employing purified non-myocytes shown in Figure 2b were limited to comparison of the characteristics of $[Ca^{2+}]_i$ signals to peptides agonists with those data obtained using purified myocytes. Thus, the elucidation of morphological characteristics and the comparison of $[Ca^{2+}]_i$ signaling data assured that the responses obtained in the present study indeed arise due to responses to agonists from the myocyte-rich preparation.

Table 1

Distinguishing cardiomyocytes from non-myocyte (fibroblast) under light microscope

	MYOCYTE	NON-MYOCYTE
Beating	**Yes**	**No**
Cytopalsm	**thick, dark**	**thin, phase-luscent**
Nucleus	**round, small**	**oval, larger**
Nucleolus	**1 or 2 (10%)**	**2 or more**
	Mayer's haem alum-eosin staining	
Beating	**no**	**no**
Cytopalsm	**red**	**pale blue**
Nucleus	**spherical, small**	**oval, larger**
Nucleolus	**1 or 2**	**several**

(Lau et al., 1980; Simpson and Savion, 1982)

Figure 2a

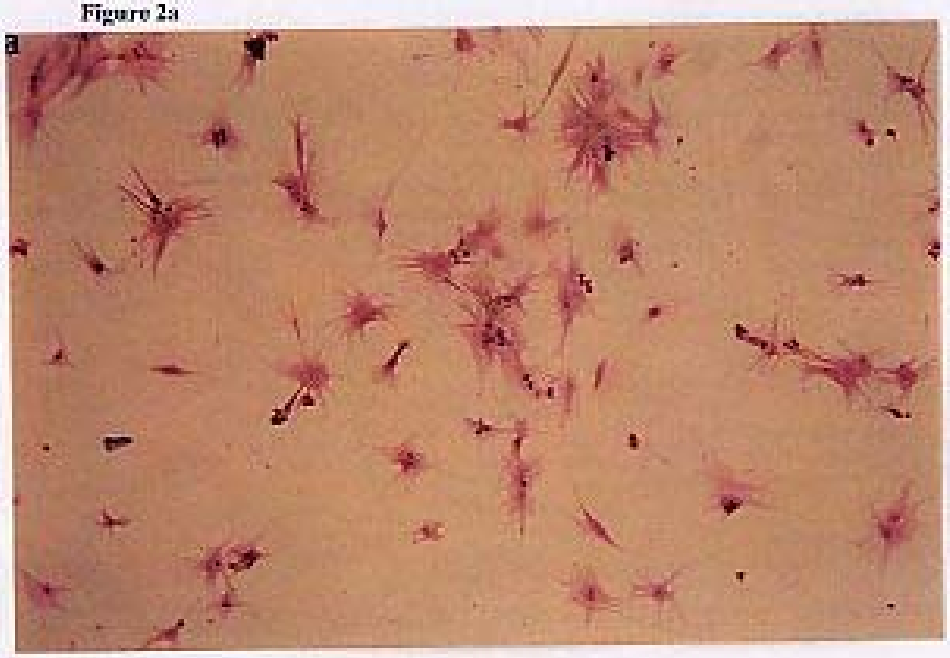


Figure 2b

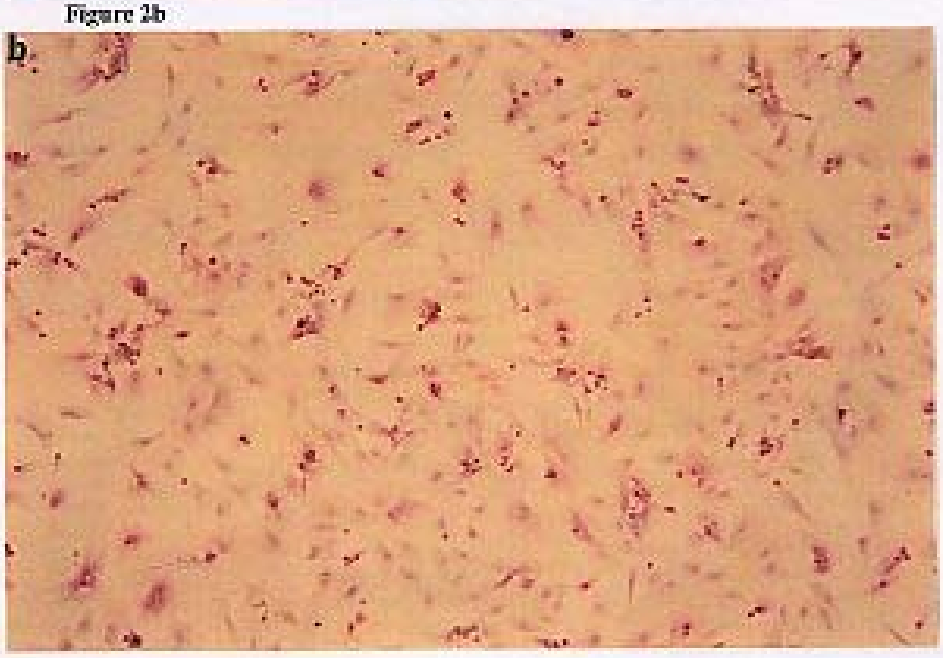
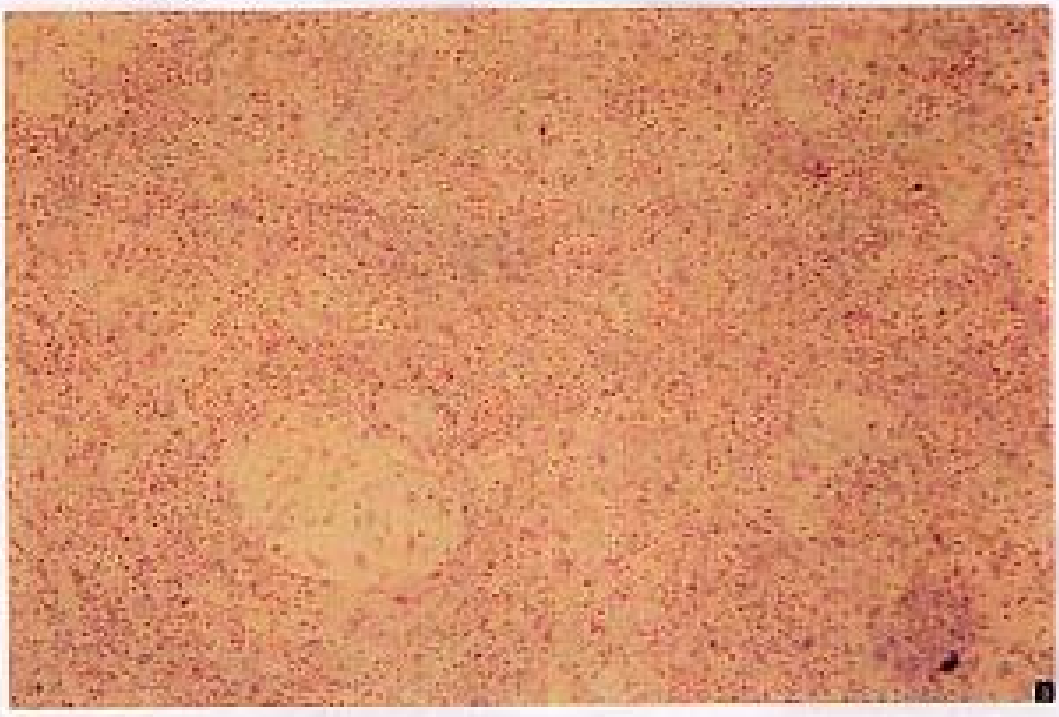


Figure 2c

Figure 2d



4.1.2 Comparison of $[Ca^{2+}]_i$ data for adherent and dispersed purified myocytes

The mean basal or resting $[Ca^{2+}]_i$ values, as determined by the Fura-2 fluorescence (25°C) in the present study varied between 35 - 74 nM and 78 - 135 nM for adherent and dispersed myocyte preparations respectively. Thus, the observed mean ± SEM resting $[Ca^{2+}]_i$ levels were significantly lower ($p < 0.05$) in adherent cardiomyocytes (56 ± 8 nM) compared to data obtained using dispersed cell suspensions (99 ± 8 nM). Since Fura-2 fluorescence data obtained using cell suspensions yielded consistent and reproducible data from different batches, it was utilized in the determination of concentration-peak $[Ca^{2+}]_i$ response relationship for the present study. On the other hand, except that adherent cells yielded quantitatively lower basal and peak $[Ca^{2+}]_i$ responses to agonists, there were no qualitative differences between responses obtained using adherent cells and dispersed myocyte suspensions.

4.1.3 The Basal $[Ca^{2+}]_i$ levels in NRC

The basal $[Ca^{2+}]_i$ levels in normal Ca^{2+} buffer was 99 ± 8 nM in multiple cells (n=54) and 121 ± 18 nM in single cells (n=23) and the differences in resting levels observed by both methods were not significant (Table 2). None of the interacting agents (AA861, MK571, MK591, BAY u9773, 2-APB, losartan or PD123319) affected the basal fura-2 fluorescence.

Table 2. Comparison of the basal $[Ca^{2+}]_i$ levels measured in the normal ($[Ca^{2+}]_e$ 1.8 mM) or Ca^{2+} free bufer ($[Ca^{2+}]_e$ 0 mM + 1.0 mM EGTA) by different methods determined using ASMC and NRC.

	Cell suspension Measurement $[Ca^{2+}]_e$		Single Cell Measurement $[Ca^{2+}]_e$
	1.8 mM	0 mM + 1.0 mM EGTA	1.8 mM
Cells			
ASMC			
WKY	97 ± 12	70 ± 8	112 ± 14
SHR	135 ±13 **	95 ± 7 **	148 ± 16
NRC			
	99 ± 8	70 ± 7	121 ± 18

*P<0.05, ** P<0.01 compared to respective groups in WKY. * P<0.05, ** P<0.01 compared to the respective groups in the absence of extracelular calcium. (**0 mM + 1.0 mM EGTA**).

4.1.4 Effect of 5-LO inhibitor, AA861, and FLAP inhibitor, MK591 on Ang II evoked increase in $[Ca^{2+}]_i$ levels in NRC

A representative tracing of Ang II evoked increases in fura-2 fluorescence ratio in the presence or absence of AA861 (10 μM) is shown (Fig 3 A & B). Addition of Ang II led to a rapid concentration-dependent increase in the ratio of fura-2 fluorescence in multiple cells with maximal increases observed between 30 and 45 seconds; at 2 min after stimulation, the fluorescence ratio decreased to a steady state above the basal level. The

CR curves to Ang II determined either in normal Ca^{2+} (1.8 mM) or Ca^{2+} free buffer is shown (Figure 4). Addition of AA861 (10 μM), or MK591(100nM) led to a significant reduction in Ang II evoked increases in peak $[Ca^{2+}]_i$ values ($P < 0.01$) in both normal and Ca^{2+} free buffer (Figure 4). Moreover, the addition of AA861 did not affect either the time to attain the peak response or the time for reduction in peak $[Ca^{2+}]_i$ to steady state level for varying concentrations of Ang II (Figure 3B). The effect of blockade on Ang II responses was similar when AA861 was increased to 30 μM. Although the E_{max} values for Ang II were relatively lower in Ca^{2+} free medium, both AA861 and MK591 evoked a similar degree of blockade (% reduction in E_{max}) of Ang II responses (right panel Figure 4). These data demonstrate that both AA861 and MK591 decrease the E_{max} with no change in EC_{50} values for Ang II-evoked peak $[Ca^{2+}]_i$ responses (Table 3), suggesting that 5-LO derived leukotrienes contribute to Ang II evoked increases in peak $[Ca^{2+}]_i$ levels via promoting the release of Ca^{2+} from intracellular store.

Table 3. Analyses of Ang II (10 pM – 10 μM), AVP (100 pM – 10 μM) and ET-1 (10 pM – 100 nM) evoked increases in peak $[Ca^{2+}]_i$ either in the presence or absence of the 5-lipoxygenase (5-LO) inhibitor, AA861 (10 or 30 μM), or $CysLT_1$ selective antagonist, MK571 (100 nM) in NRC maintained at 37°C.

	Ca^{2+} 1.8 mM		**Ca^{2+} 0 mM + 1.0 mM EGTA**	
Agonist	**EC_{50} (nM)**	**E_{max} (nM)**	**EC_{50} (nM)**	**E_{max} (nM)**
ANG II				
Control	12.6 ± 4.6	498 ± 30	13.5 ± 3.0	337 ± 20
AA861 10 μM	13.9 ± 6.1	344 ± 15**	15.3 ± 4.1	230 ± 15**
AA861 30 μM	15.1 ± 3.5	313 ± 28**	16.6 ± 4.5	195 ± 11**
MK571 100 nM	16.8 ± 7.2	370 ± 22**	18.4 ± 6.1	236 ± 24**
AVP				
Control	18.9 ± 7.3	210 ± 11		
AA861 30 μM	21.5 ± 6.6	199 ± 14		
MK571 100 nM	20.4 ± 7.8	212 ± 13		
ET-1				
Control	6.3 ± 1.4	253 ± 18		
AA861 30 μM	7.2 ± 2.1	232 ± 21		
MK571 100 nM	8.4 ± 2.3	228 ± 19		

Values shown are means ± SE of eight experiments. The basal level $[Ca^{2+}]_i$ was 90 ± 12 nM in normal Ca^{2+} buffer. AA861 or MK571 was maintained in the cuvette in the indicated buffer for a period of 3 min prior to the addition of the agonist. The E_{max} to Ang II or AVP were attained at 1 μM whereas the E_{max} to ET-1 was reached at 100 nM. *Note*: Stimulation with either AVP or ET-1 was not performed in Ca^{2+} free buffer since no significant changes in EC_{50} and E_{max} values were noted in normal Ca^{2+} buffer. ** $P < 0.01$ compared to respective control group.

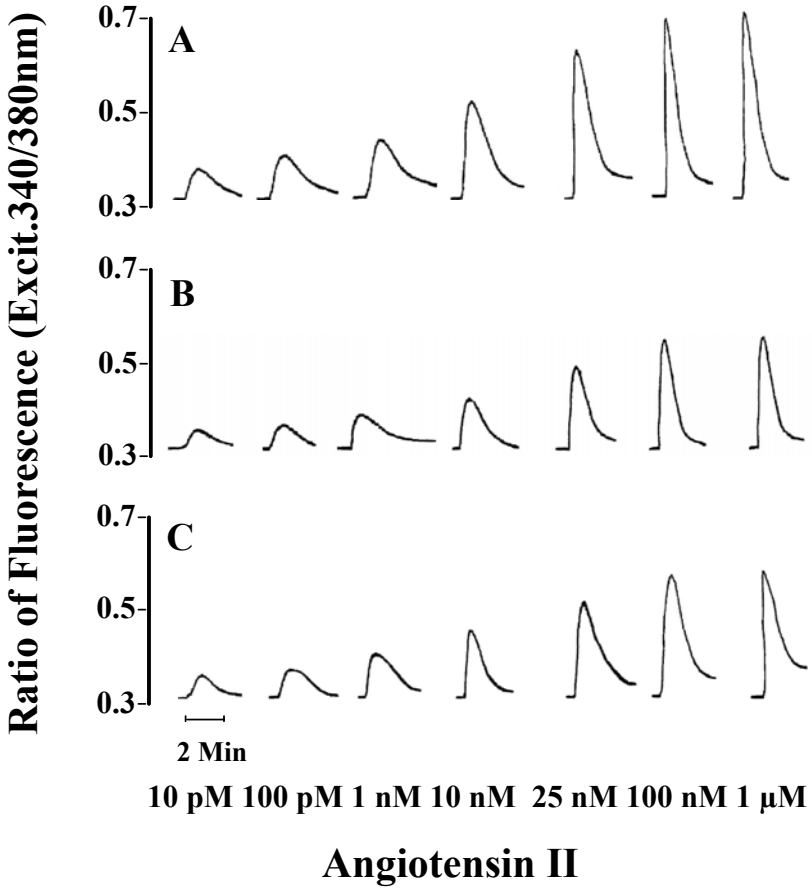


Figure 3. A representative experiment of angiotensin II (Ang II) evoked changes in the ratio of fura-2 fluoresence (340/380 nm) in neonatal rat cardiomyocytes (NRC). Primary cultures of NRC loaded with fura-2 were stimulated with increasing concentrations of Ang II (10 pM – 1 μM) either in the absence (**A – Control**) or the presence of AA861 (10 μM; **B** – middle panel) or MK571 (100 nM; **C** – lower panel). These agents were maintained in the cuvette in Krebs buffer (pH 7.4) containing normal Ca^{2+} (1.8 mM) at 37^{o}C for 3 min. prior to challenge with indicated concentration of Ang II. Note: Each sample with fura-2 loaded NRC was stimulated only once with a single concentration of Ang II.

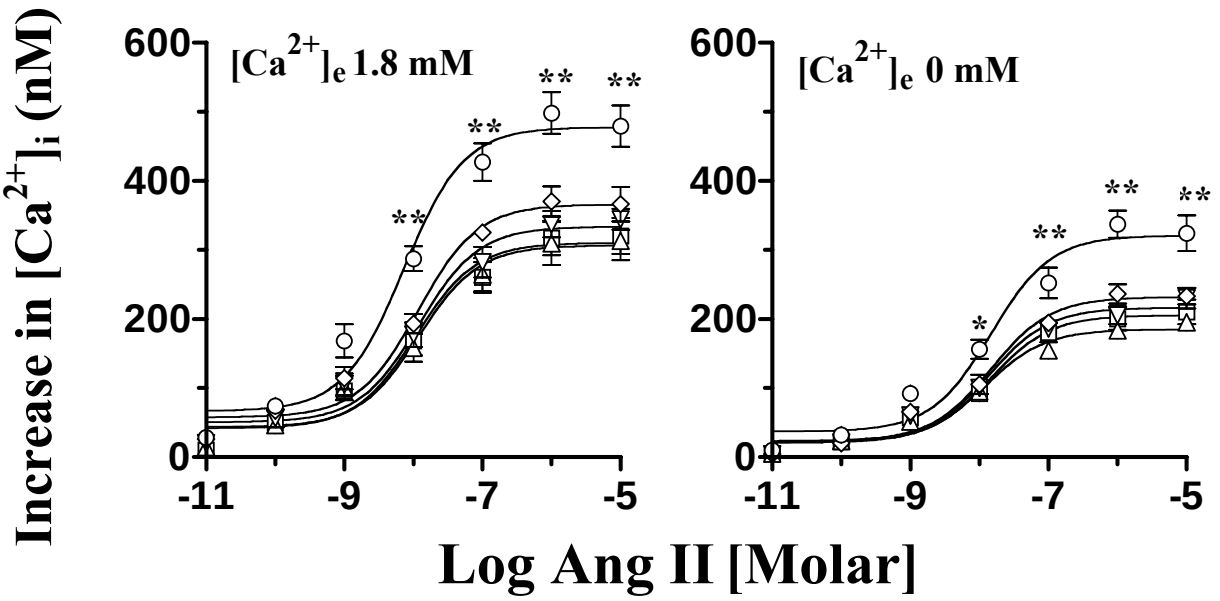


Figure 4. The effects of 5-lipoxygenase (5-LO) inhibitor, AA861, and $CysLT_1$ selective antagonist, MK571, on peak $[Ca^{2+}]_i$ responses to Ang II in NRC. Primary cultures of NRC were stimulated with increasing concentrations of Ang II either in the presence or in the absence (Control;○) of AA861 (10 μM, ▽**),** or MK591 (100nM; □) or MK571(100 nM; ◊), or BAY u9773 (100nM, Δ) in Krebs buffer (pH 7.4) at 37°C. Ang II concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with either Ca^{2+} being present (1.8 mM; left panel) or absent (0 mM Ca^{2+} + 1 mM EGTA, pH adjusted to 7.4; right panel) in the buffer. Each CR curve was determined eight times using different batches of NRC. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to AA861, MK591, MK571 and Bay u9773 treated cells.

4.1.5 Effect of $CysLT_1$ receptor antagonist, MK571, and dual $CysLT_1$/$CysLT_2$ receptor antagonist, Bay u9773 on Ang II evoked increase in $[Ca^{2+}]_i$ levels in NRC

A representative tracing of Ang II evoked increases in fura-2 fluorescence ratio in the presence or absence of MK571 (100 nM) is shown (Fig 3C). The effect of $CysLT_1$ receptor antagonist, MK571, and dual $CysLT_1$/ $CysLT_2$ receptor antagonist, Bay u9773 on Ang II evoked increase in $[Ca^{2+}]_i$ levels in NRC is shown (Figure 4). Addition of MK571 (100 nM), or Bay u9773 (100nM) led to a significant reduction in Ang II evoked increases in peak $[Ca^{2+}]_i$ values ($P < 0.01$) in both normal and Ca^{2+} free buffer. Although the E_{max} values for Ang II were relatively lower in Ca^{2+} free medium, both MK571 and Bay u9773 evoked a higher degree of blockade (% reduction in E_{max}) of Ang II responses (right panel Figure 4). Furthermore, in both normal and Ca^{2+} free buffer, Bay u9773 caused a higher degree of blockade of Ang II responses than MK571 did, suggesting that both $CysLT_1$ and $CysLT_2$ receptor activation contribute to Ang II evoked increase in $[Ca^{2+}]_i$ levels in NRC.

4.1.6 Effects of AA861, MK591, MK571 and Bay u9773 on AVP and ET-1 evoked increase in $[Ca^{2+}]_i$ levels in NRC

Both AVP and ET-1 evoked concentration-dependent increases in $[Ca^{2+}]_i$ levels in NRC (Figure 5). The EC_{50} and E_{max} values are summarized in Table 3. High concentration

of either AA861 (30 μM), or MK591 (100nM), or MK571 (100 nM) failed to affect the concentration-peak $[Ca^{2+}]_i$ response curves to AVP or ET-1 (Figure 5).

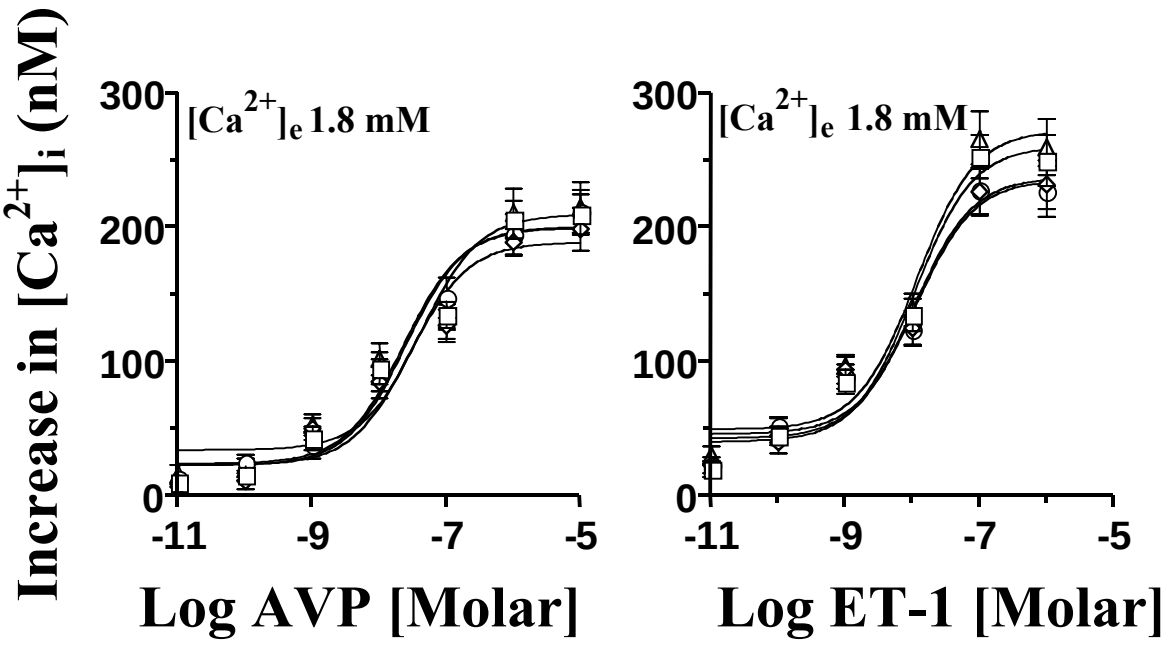


Figure 5. The effects of 5-lipoxygenase (5-LO) inhibitor, AA861, $CysLT_1$ selective antagonist, MK571, and dual $CysLT_1$ / $CysLT_2$ antagonist, Bay u9773 on peak $[Ca^{2+}]_i$ responses to AVP (Left panel) and ET-1 (Right panel) in NRC. Primary cultures of NRC were stimulated with increasing concentrations of AVP or ET-1 either in the presence or in the absence (Control; □) of AA861 (30 μM; ◊), or MK571 (100 nM; ○), or Bay u9773 (100nM; Δ) in Krebs buffer (pH 7.4) at 37°C. AVP and ET-1 concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being present (1.8 mM) in the buffer. Each CR curve was determined six times using different batches of NRC.

4.1.7 Effects of AT_1 antagonist, losartan, AT_2 antagonist, PD123319, and $InsP_3$ antagonist, 2APB on Ang II evoked increase in $[Ca^{2+}]_i$ levels in NRC

In both normal and Ca^{2+} free buffer, the AT_1 selective antagonist, losartan, abolished the responses to Ang II, whereas PD123319 had no effect on Ang II evoked peak $[Ca^{2+}]_i$ responses (Figure 6). Moreover, the $InsP_3$ blocker, 2-APB, significantly attenuated Ang II evoked peak $[Ca^{2+}]_i$ responses in both normal and Ca^{2+} free buffer (Figure 6).

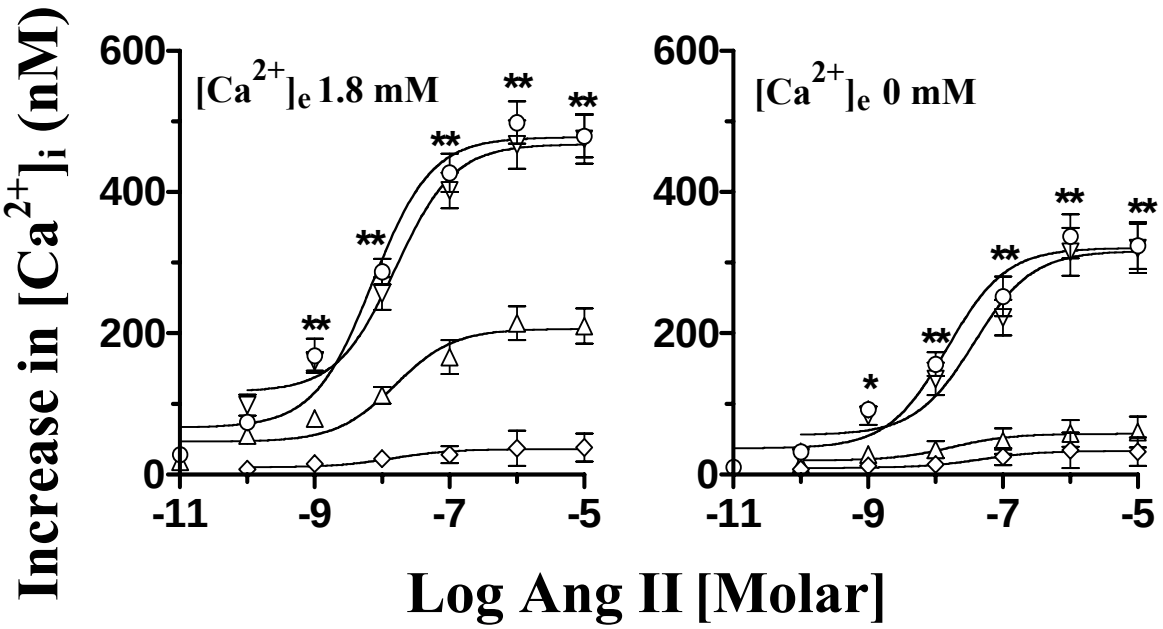


Figure 6. The effects of AT_1 receptor antagonist, losartan, AT_2 receptor antagonist, PD123319, and $InsP_3$ receptor antagonist, 2-APB on peak $[Ca^{2+}]_i$ responses to Ang II in NRC. NRC were stimulated with increasing concentrations of Ang II either in the absence (Control; O) or in the presence of losartan (1.0 μM; ◊), or PD123319 (1.0 μM; ∇), or 2APB (5 μM; Δ) in Krebs buffer (pH 7.4) at 37°C. Ang II concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being present (1.8 mM, left panel) or absent (0 mM, right panel) in the buffer. Each CR curve was determined eight times using different batches of NRC. $^{**}P < 0.01$ compared to the losartan and 2-APB treated cells.

4.1.8 Both LTD_4 and LTC_4 evoked concentration-dependent increase in $[Ca^{2+}]_i$ levels in NRC

A representative tracing of Ang II, LTD_4 and LTC_4 evoked $[Ca^{2+}]_i$ response is shown (Figure 7). In both normal and Ca^{2+} free buffer, both LTC_4 and LTD_4 evoked rapid increases in peak $[Ca^{2+}]_i$ levels (E_{max}: LTD_4>>LTC_4) with a similar time course but much lower E_{max} in comparison to Ang II. The peak responses were attained at 40, 43 and 47 seconds after the addition of Ang II, LTD_4 and LTC_4 respectively (Figure 7). The plateau phase that sustained at levels slightly above their respective baseline values were reached between 120 and 180 seconds after the addition of respective agonist(s). There were no significant differences between them (Figure 7). The E_{max} values for both LTD_4 and LTC_4 in normal Ca^{2+} buffer were not significantly different from the ones observed in Ca^{2+} free buffer (Table 4 & Figure 8 & 9).

Table 4. Analyses of LTD_4 (10 pM – 10 μM) and LTC_4 (10 pM – 10 μM) evoked increases in $[Ca^{2+}]_i$ either in the presence or absence of the $CysLT_1$ selective antagonist, MK571 (100 nM), or the dual CysLT antagonist, Bay u9773 (100 nM), in adherent NRC maintained at 37°C.

Agonist	**EC_{50} (nM)**	**E_{max} (nM)**
LTD_4		
Control	17.0 ± 4.5	217 ± 18
MK571 (100 nM)	37.3 ± 5.6*	52 ± 11**
Bay u9773 (100 nM)	57.5 ± 7.4*	17 ± 7**
LTC_4		
Control	49.8 ± 16.5	107 ± 10
MK571 (100 nM)	42.6 ± 17.3	95 ± 12
Bay u9773 (100 nM)	63.8 ± 6.2	21 ± 9**

Values shown are means ± SE of six experiments. The basal level of $[Ca^{2+}]_i$ in the absence of either LTD_4 or LTC_4 stimulation was 90 ± 12 nM. MK571 or Bay u9773 was added 3 min prior to the addition of agonist to the cuvette. * $P < 0.05$, ** $P < 0.01$ compared to respective control groups.

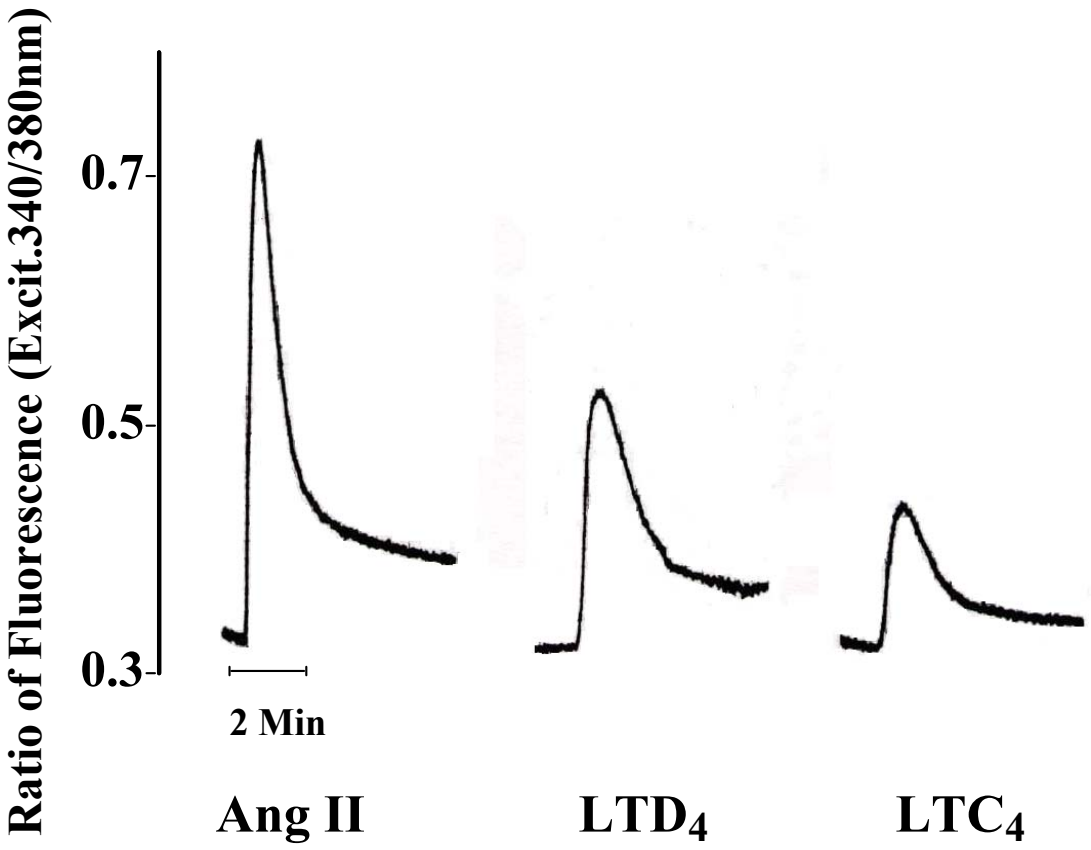


Figure 7. A representative experiment of Ang II, LTD_4 and LTC_4 evoked changes in the ratio of fura-2 fluoresence (340/380nm) in NRC. Primary cultures of NRC loaded with fura-2 were stimulated with either Ang II (100 nM), or LTD_4 (100 nM) or LTC_4 (100 nM). These agents were added to the cuvette in Krebs buffer (pH 7.4) containing normal Ca^{2+} (1.8 mM) at 37^{o}C. Note: Each sample containing fura-2 loaded NRC was stimulated only once with a single agonist.

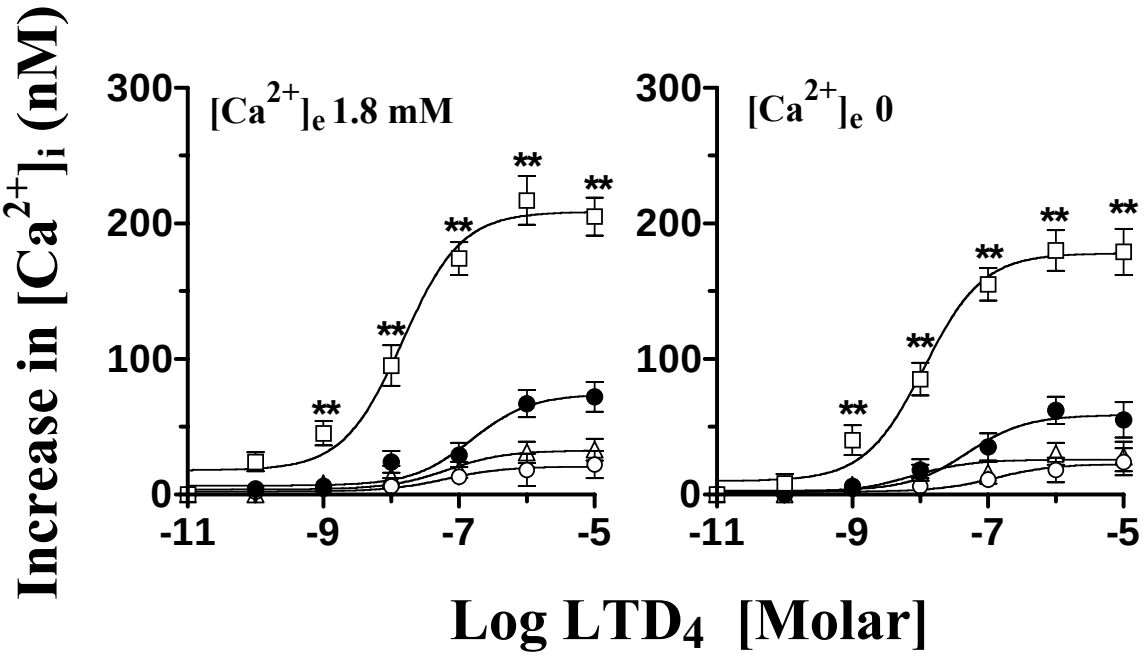


Figure 8. The effects of $CysLT_1$ selective antagonist, MK571, dual $CysLT_1$ / $CysLT_2$ antagonist, Bay u9773, and $InsP_3$ receptor antagonist, 2-APB on peak $[Ca^{2+}]_i$ responses to LTD_4 in NRC. NRC were stimulated with increasing concentrations of LTD_4 either in the absence (Control;□) or in the presence of MK571 (100 nM; ●), or Bay u9773 (100 nM; ○), or 2-APB (5 μM; Δ) in Krebs buffer (pH 7.4) at 37°C. LTD_4 concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being present (1.8 mM, left panel) or absent (0 mM Ca^{2+}+ 1.0 mM EGTA, right panel) in the buffer. Each CR curve was determined six to eight times using different batches of NRC. $^{**}P < 0.01$ compared to the MK571, Bay u9773 and 2-APB treated cells group.

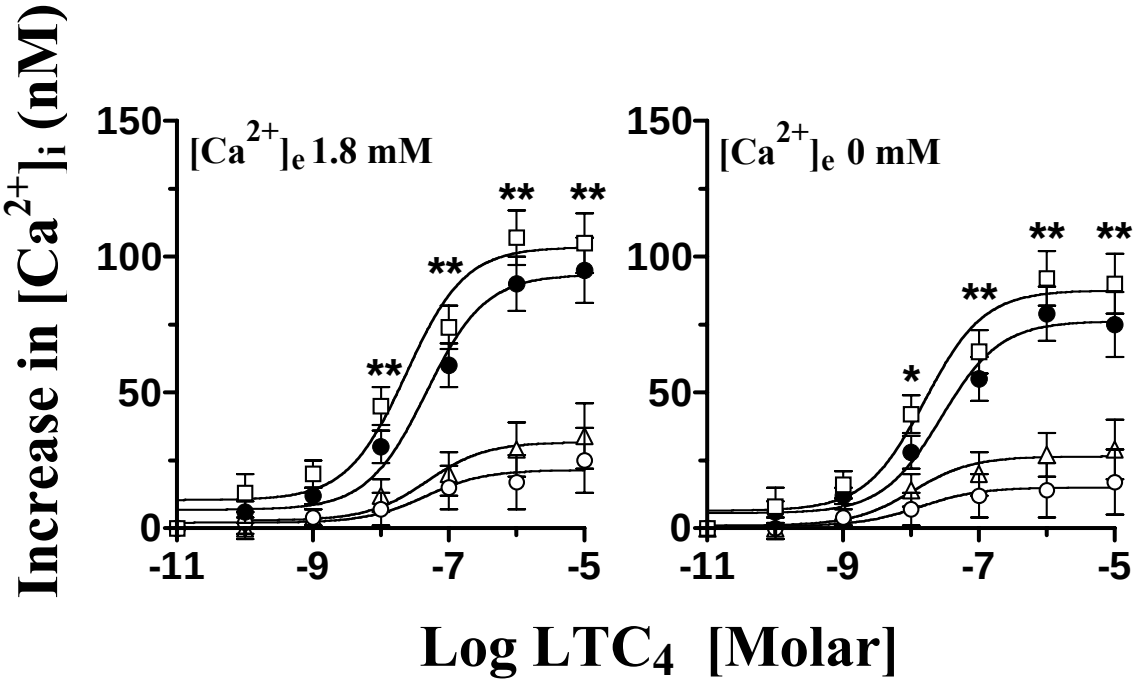


Figure 9. The effects of $CysLT_1$ selective antagonist, MK571, dual $CysLT_1$ / $CysLT_2$ antagonist, Bay u9773, and $InsP_3$ receptor antagonist, 2-APB on peak $[Ca^{2+}]_i$ responses to LTC_4 in NRC. NRC were stimulated with increasing concentrations of LTC_4 either in the absence (Control;□) or in the presence of MK571(100 nM; ●), or Bay u9773 (100 nM; ○), or 2-APB (5 μM; Δ) in Krebs buffer (pH 7.4) at 37°C. LTC_4 concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being present (1.8 mM, left panel) or absent (0 mM Ca^{2+} + 1.0 mM EGTA, right panel) in the buffer. Each CR curve was determined six to eight times using different batches of NRC. $^{**}P < 0.01$ compared to the Bay u9773 and 2-APB treated cells group.

4.1.9 Effect of $CysLT_1$ receptor antagonist, MK571, dual $CysLT_1/CysLT_2$ receptor antagonist, Bay u9773, and $InsP_3$ receptor antagonist, 2-APB on both LTD_4 and LTC_4 evoked increase in $[Ca^{2+}]_i$ levels in NRC

Inclusion of $CysLT_1$ receptor antagonist, MK571, significantly decreased the E_{max} and increased the EC_{50} value for LTD_4, but it failed to evoke a significant reduction in the weak $[Ca^{2+}]_i$ signals evoked by LTC_4 in either normal or Ca^{2+} free buffer (Figure 8 & 9). However, inclusion of Bay u9773 abolished both LTD_4 and LTC_4 evoked $[Ca^{2+}]_i$ response in either normal or Ca^{2+} free buffer (Figure 8 & 9). The analysis of data obtained from several CR curves is summarized in Table 4. Furthermore, the $InsP_3$ blocker, 2-APB, completely blocked both LTD_4 and LTC_4 evoked peak $[Ca^{2+}]_i$ responses in both normal and Ca^{2+} free buffer (Figure 8 & 9).

4.1.10 Effect of 5-LO inhibitor, AA861, and $CysLT_1$ antagonist, MK571 on Ang II evoked increase in $[Ca^{2+}]_i$ levels in single NRC

Single cell Ca^{2+} imaging studies revealed qualitatively similar results. A typical experiment performed with Ang II stimulation (50 nM) on three different coverslips loaded with fura-2 on the same day using the same batch of cardiomyocytes is shown (Figure 10). Basal fluorescence levels prior to stimulation are shown in the top panel

[control – (A), AA861 – (B), MK571 - (C)]. The lower panel shows the effect of Ang II at 30 seconds in the presence of either AA861 (E) or MK571 (F). The responses are compared with images determined for Ang II in the absence of these interacting agents [control – (D)]. Single cell fluorescence determination from several experiments (n = 5) gave the following absolute $[Ca^{2+}]_i$ values: control - 585 ± 34 nM; AA861 - 400 ± 27 nM ($P < 0.01$); MK571 - 460 ± 21 nM ($P < 0.01$). Although single cell E_{max} values for Ang II were higher when compared to data obtained with multiple cells, a similar pattern of blockade in the presence of AA861 or MK571 was evident. Between 2 and 3 min intervals the cells had reached steady state fluorescence close to the basal value suggesting the fluorescence changes are consistent with $[Ca^{2+}]_i$ changes and that the results gathered were not due to photobleaching (data not shown).

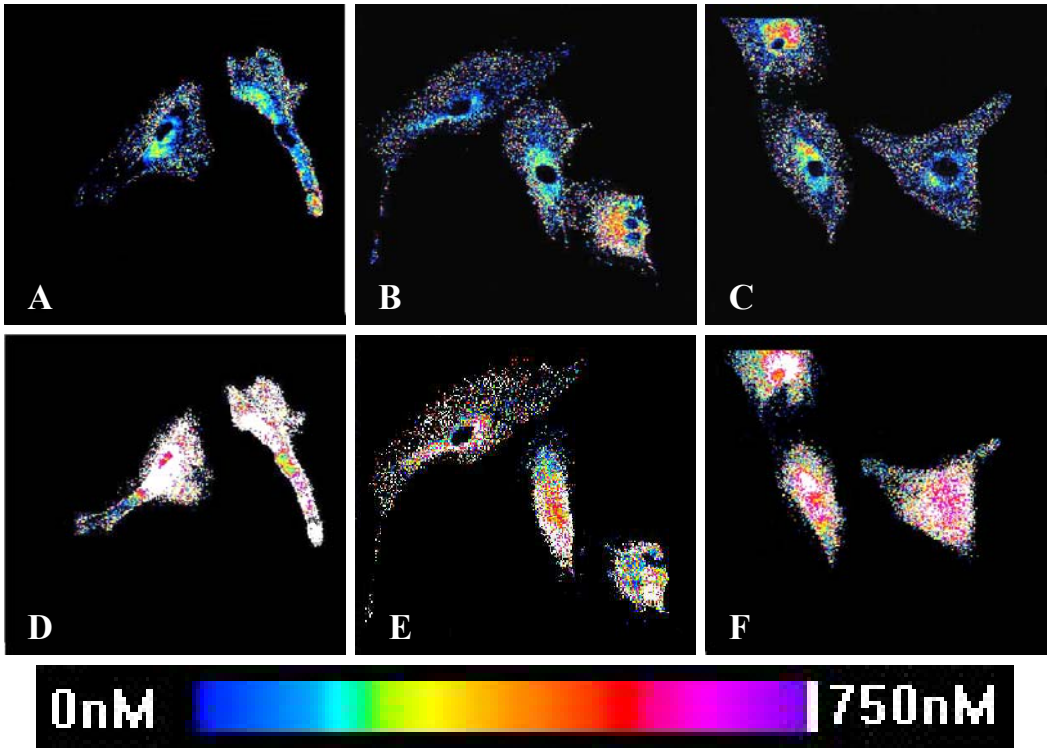


Figure 10. Fura-2 Ca^{2+} imaging in single neonatal rat cardiomyocyte stimulated with Ang II (50 nM). Ca^{2+} images were acquired at a rate of 3 images per second, using three different coverslips loaded with fura-2 under identical conditions on the same day. The upper panels (**A, B, C**) depicts basal fura-2 fluorescence images (340/380 nm excitation ratio) acquired before the addition of Ang II in the absence (**A**) or the presence of AA861 (**B**) or MK571 (**C**). The lower panels show changes in fura-2 fluorescence at 30 seconds after the addition of Ang II in the same cells (**D** – Ang II alone; **E** - Ang II in the presence of AA861, 10 μM; **F** - Ang II in the presence of MK571, 100 nM). The aggregate $[Ca^{2+}]_i$ values in single cells obtained were (nM): **A** -110; **B** -124, **C** - 117; **D** - 710; **E** - 470; **F** - 530). At the end of two min. the fluorescence values were less than 200 nM in all the cells (data not shown). Similar response patterns were recorded in five separate experiments.

4.1.11 Effects of Ang II, AVP and ET-1 on total CysLTs production in NRC

The basal CysLT level in the culture medium was 9.7 ± 1.9 pg/2h/10^6 cells. Addition of Ang II led to a rapid, time-dependent increase in CysLT level reaching a maximum (19.8 ± 2.6 pg/ml/10^6 cells) at 1 min remained at the same level until 5 min and gradually decreased thereafter. However, the increases in CysLT levels were significantly higher (15.7 ± 1.8 pg/ml/10^6 cells) even 30 seconds after the addition of Ang II (Figure 11). In contrast, neither AVP (100nM) nor ET-1 (100 nM) evoked a time–dependent increase in CysLT levels (Figure 11).

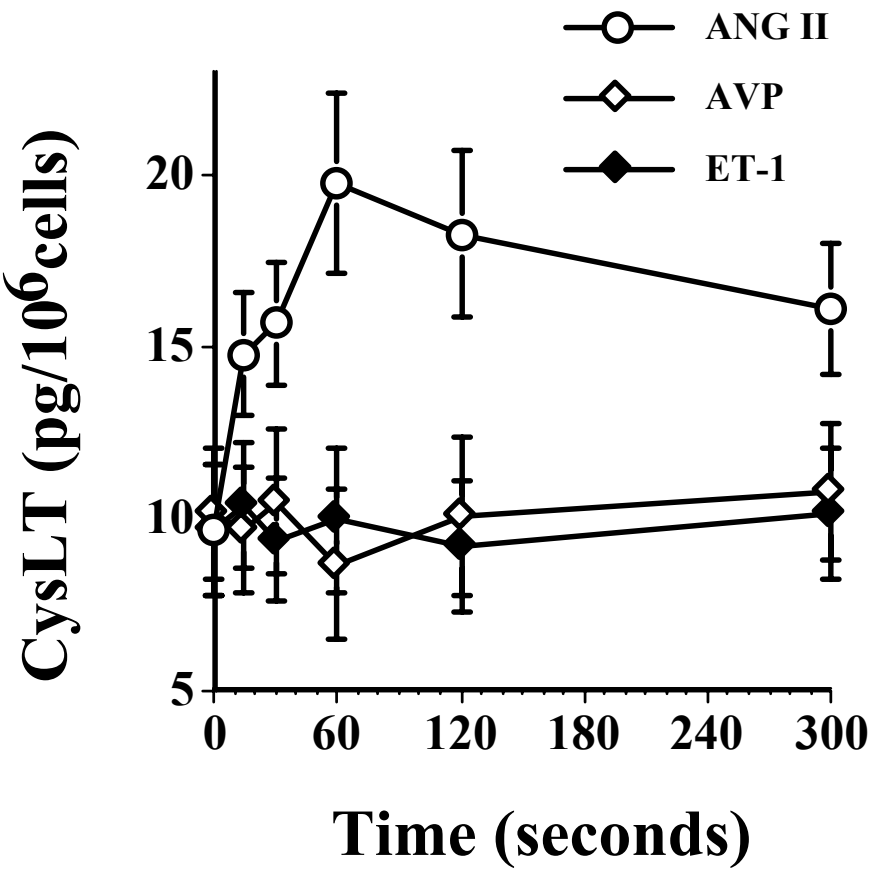


Figure 11. The time dependence of Ang II evoked increases in CysLT levels in the culture medium of NRC. Total CysLT levels in the medium at different time points following stimulation with a fixed concentration of Ang II (100 nM;○), AVP (100 nM; ✧) and ET-1(100 nM; ◆) were shown. Each curve was determined five times using different batches of NRC.

4.1.12 Effects of AA861, MK591, losartan and PD123319 on Ang II evoked total CysLTs production

The CR determinations for CysLT generation revealed that the Ang II effect was also concentration-dependent (Figure 12). The E_{max} for Ang II (100 nM) evoked CysLT release was two fold higher than the basal level. In contrast to Ang II, addition of increasing concentration of either ET-1 or AVP failed to evoke a significant increase in CysLT production (Figure 12). Inclusion of losartan, or AA861, or MK591 abolished Ang II-evoked total CysLT release into the culture medium whereas addition of PD123319 failed to affect Ang II evoked increases in CysLT production (Figure 12). None of the antagonists or inhibitors affected the basal total CysLT generation. CysLT generation was significantly higher at 30 seconds and the peak $[Ca^{2+}]_i$ increase was reached at 40 seconds after the addition of Ang II (100 nM) suggesting that the increased CysLT generation evoked by Ang II may contribute to elevation in $[Ca^{2+}]_i$ (Figure **7** and Figure 11).

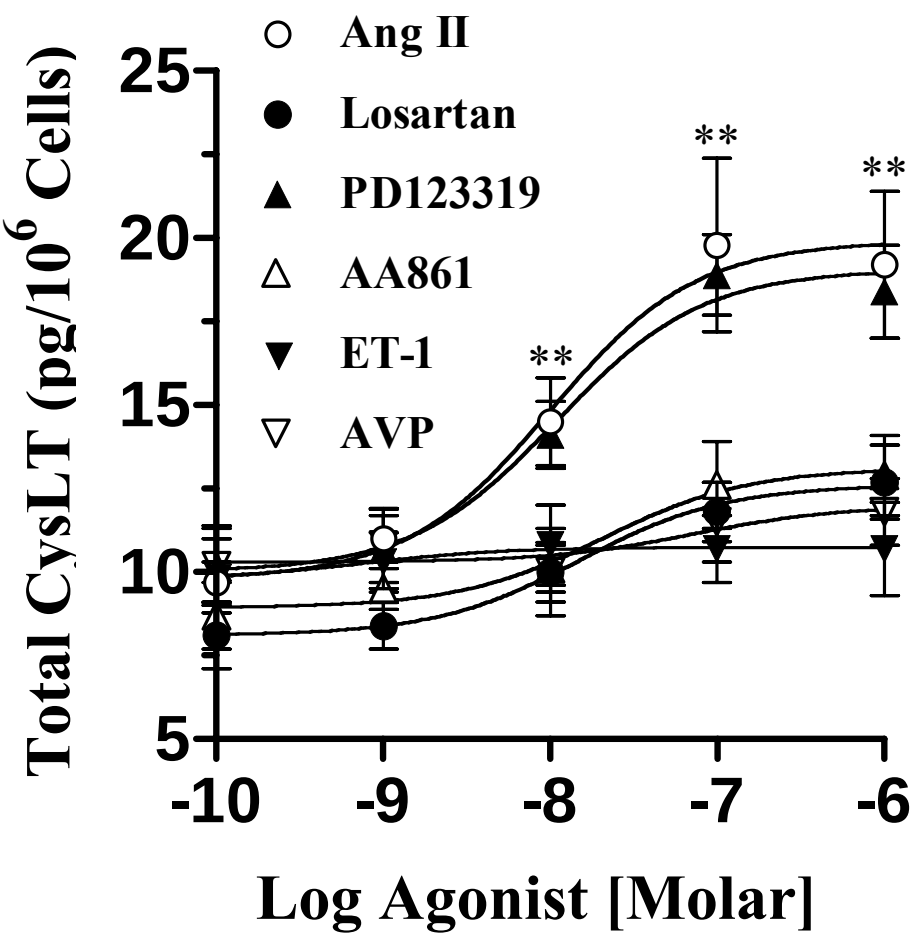


Figure 12. The effects of losartan, PD123319 and AA861 on Ang II–evoked increases in CysLT levels and the lack of effect of ET-1 and AVP on CysLT production in NRC. Line graphs provide a comparison of elevation in total CysLT levels attained in the medium one min. after stimulation with increasing concentrations of Ang II either in the presence or in the absence (Control: Ang II alone; ○) of losartan (1 μM; ●), PD123319 (1 μM; σ), or AA861 (10 μM; Δ). Moreover, the lack of significant changes in CysLT levels in the medium 1 min after stimulation with increasing concentrations of either AVP or ET-1 are also shown. Each curve was determined five times using different batches of NRC. ** $P < 0.01$ compared with losartan, AA861, AVP, and ET-1 treated cells.

4.1.13 $CysLT_1$ and $CysLT_2$ receptor mRNA expression in NRC

Both $CysLT_1$ and $CysLT_2$ receptor mRNA was detected in NRC by northern hybridization analysis (Figure 13). It appeared that the mRNA of $CysLT_1$ was more abundantly expressed in NRC than that of $CysLT_2$. However, the precise quantilation for the expression of $CysLT_1$ and $CysLT_2$ need to be determined by employing other experimental methods.

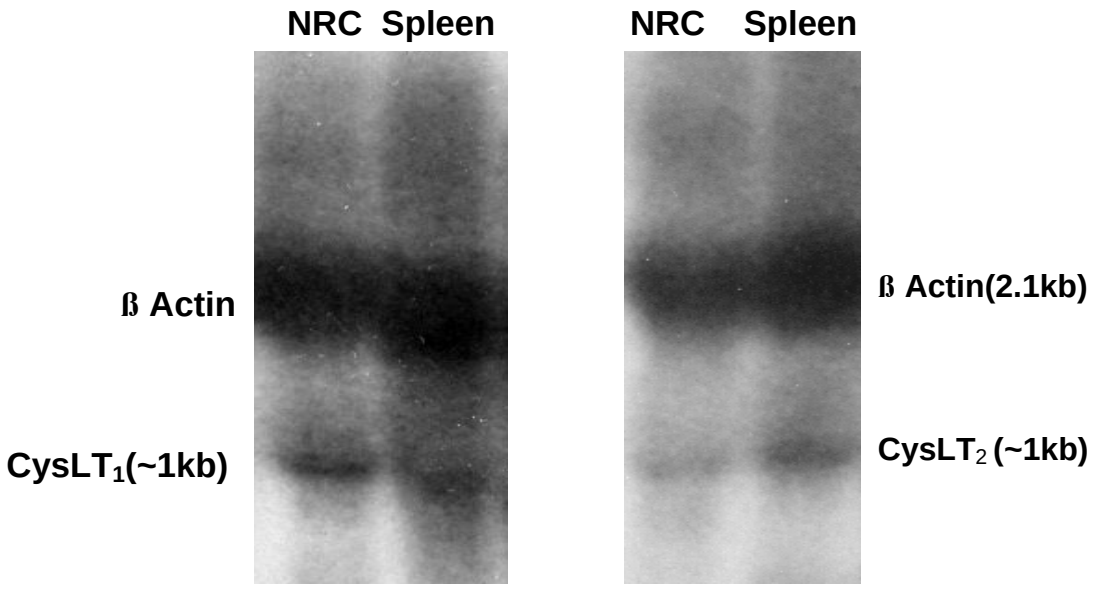


Figure 13. $CysLT_1$ and $CysLT_2$ mRNA expression in primary cultures of NRC. Spleen total RNA isolated from SD rats was used as a positive control.

4. 2 Results from Studies Using ASMC

4.2.1 Aortic smooth muscle cells

After initial isolation, the time required for ASMC to reach confluency in the flask varied from 6 to 8 days. ASMC were allowed to proliferate due to the presence of L-glutamine, horse serum, and newborn calf serum in the incubation medium. ASMC grew in a characteristic 'Hill and Valley' pattern, with the valleys representing the monolayer of cells. Individual ASMC are spindle shaped and they differ from fibroblasts in that they have only one or two nucleoli present in the nucleus while fibroblasts have four or five. Although cobble-shaped EC's are common contaminants of the culture, care was taken to ensure the meticulous removal of these cells during the initial isolation as well as during the period of initial growth before the cells attained confluency. Although ASMC could be more adequately characterized with the routine use of anti- α-actin or anti-myosin antibodies, the above procedure was used to obtain ASMC cultures with high purity. The efficacy of this technique was verified with occasional antibody studies. Viability of cells was repeatedly monitored using the trypan blue exclusion test, whereby dead or dying cells take up the blue dye, while healthy cells remain intact. Viability of cells was consistently greater than 90%. ASMC viewed under a microscope on both low and high power are shown in Figure14.

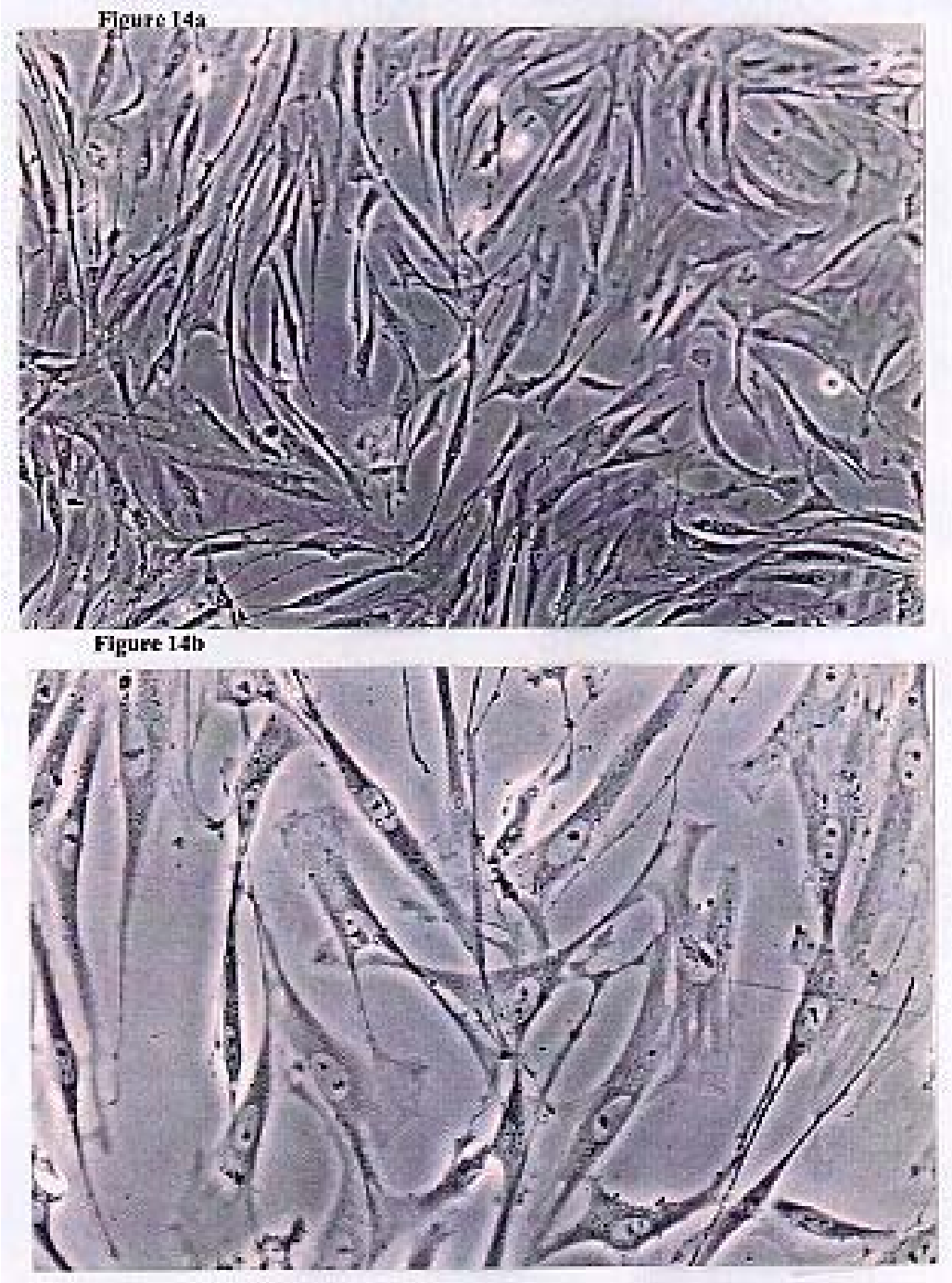


Figure 14. Aortic smooth muscle cells (ASMC) when viewed under a microscope at (**a**) x 10 and (**b**) x 40 magnification. Spindle shaped ASMC have one or two nucleoli-clearly observed at the higher magnification.

4.2.2 Basal systolic blood pressures(SBP) and basal $[Ca^{2+}]_i$ levels in ASMC of WKY and SHR

Prior to the experiments, SBP of SHR and WKY rats were measured using rat tail cuff using BP monitor/amplifier and rat tail occluder cuff (Harvard Apparatus, St Laurent, Quebec). The baseline SBP before the isolation of ASMC was 112± 7 mmHg and 166 ± 8 mmHg in WKY and SHR (n=17 for each strain, $p<0.01$) respectively. The basal $[Ca^{2+}]_i$ levels in normal Ca^{2+} buffer were (nM) 97 ± 12 (WKY), 135 ± 13 (SHR) in multiple cells (n=26) and 106 ± 12 nM (WKY), 148 ± 16 nM (SHR) in single cells (n=18) (Table 2). The differences in basal $[Ca^{2+}]_i$ levels observed by both methods were not significant. None of the antagonists or inhibitors affected the basal $[Ca^{2+}]_i$ levels in either strains.

4.2.3 Effects of 5-LO inhibitor, AA861, and FLAP inhibitor, MK591 on Ang II evoked increase in $[Ca^{2+}]_i$ levels in ASMC of WKY and SHR

A representative tracing of Ang II evoked increases in fura-2 fluorescence ratio in the presence or absence of MK591 (100 nM) is shown (Figure 15). In both WKY and SHR, Ang II (100 nM) evoked a rapid concentration-dependent increase in the ratio of fura-2 fluorescence with maximal increases observed around 45 seconds that was followed by a decrease till reaching a steady state above the basal level at 2 min after stimulation (Figure 15). The E_{max} values for Ang II evoked increases in $[Ca^{2+}]_i$ in ASMC were significant higher in SHR than that in WKY (Table 5). In normal Ca^{2+} (1.8 mM) buffer,

either AA861 (10 μM), or MK591 (100nM) significantly reduced Ang II evoked increases in peak $[Ca^{2+}]_i$ values in both WKY ($P < 0.05$) and SHR ($P < 0.01$). The reduction in Ang II evoked responses (both the magnitude of the responses and the % reduction in E_{max} reductions) were more pronounced in SHR (WKY 12-18%; SHR 25-39%) as shown (Figure 16 & Table 5). Similar effects were observed in Ca^{2+} free buffer, except the lower E_{max} values for Ang II and higher percentage reduction in E_{max} values (WKY 17-24%; SHR 34-49%) (Figure 17 & Table 5). Both AA861 and MK591 evoked similar degree of blockade of Ang II responses. Neither AA861 nor MK591 affected the basal fura-2 fluorescence and the time courses for either agonist to evoke the peak response or recovering to the steady state level (Figure 15). Moreover, none of them led to a significant change in EC_{50} values for Ang II.

Table 5. Analyses of angiotensin II (Ang II, 1 μM), endothelin-1 (ET-1, 100 nM) and AVP (1 μM) evoked increases in $[Ca^{2+}]_i$ either in the presence or in the absence of the different inhibitors in ASMC form WKY and SHR

	WKY		SHR		WKY		SHR	
	$[Ca^{2+}]_e$ 1.8 mM				$[Ca^{2+}]_e$ 0 mM + 1.0 mM EGTA			
Agonist	EC_{50} (nM)	E_{max} (nM)	EC_{50} (nM)	E_{max} (nM)	EC_{50} (nM)	E_{max} (nM)	EC_{50} (nM)	E_{max} (nM)
Ang II								
Control	19 ± 3	445 ± 32	21 ± 4	615 ± 34^^	17 ± 3	220 ± 15	22 ± 4	315 ± 24^^
AA861	20 ± 5	368 ± 25*	22 ± 4	375 ± 29**	16 ± 4	163 ± 13*	23 ± 5	167 ± 15**
MK571	22 ± 3	393 ±29	20 ± 4	460 ± 28**	17 ± 3	180 ± 13*	22 ± 4	210 ± 20**
Bay u9773	19 ± 5	365 ± 27*	23 ± 5	383 ± 30**	19 ± 4	165 ± 14*	20 ± 5	163 ± 17**
MK591	20 ± 4	370 ± 27*	24 ± 5	390 ± 19**	19 ± 4	170 ± 16*	18 ± 5	182 ± 18**
Losartan	ND	41 ± 10*	ND	55 ± 11**	ND	24 ± 10*	ND	32 ± 8**
PD123319	22 ± 4	415 ± 32	16 ± 54	592 ± 32	32 ± 6	210 ± 15	16 ± 54	308 ± 26
2-APB	23 ± 4	198 ± 23*	21 ± 3	256 ± 24**	17 ± 3	87 ± 11*	25 ± 3	113 ± 11**
ET-1								
Control	7 ± 1	232 ± 21	5 ± 2	326 ± 26				
AA861	7 ± 2	245 ± 25	5 ± 2	342 ± 31				
MK571	8± 2	256 ± 24	5 ± 2	353 ± 28				
Bay u9773	9 ± 2	216 ± 23	6 ± 1	347 ± 29				
AVP								
Control	24 ± 2	210 ± 18	27 ± 2	303 ± 24				
AA861	27 ± 3	200 ± 20	25 ± 4	293 ± 28				
MK571	23 ± 2	225 ± 22	25 ± 4	315 ± 28				
Bay u9773	25 ± 2	220 ± 21	32 ± 5	306 ± 27				

Values shown are mean ± SEM of six experiments. The basal levels of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of ASMC in the absence of agonist stimulation were **97 ± 12** nM and **135 ± 13** nM for WKY and SHR respectively. Either AA861 (10 μM), or MK591 (10 nM), or MK571 (100 nM), or Bay u9773(100 nM) were added for 3 min prior to the addition of agonist. * P <0.05, ** P < 0.01 compared to respective control group in the same strain rats. ** P < 0.01 compared to the control of WKY strain.

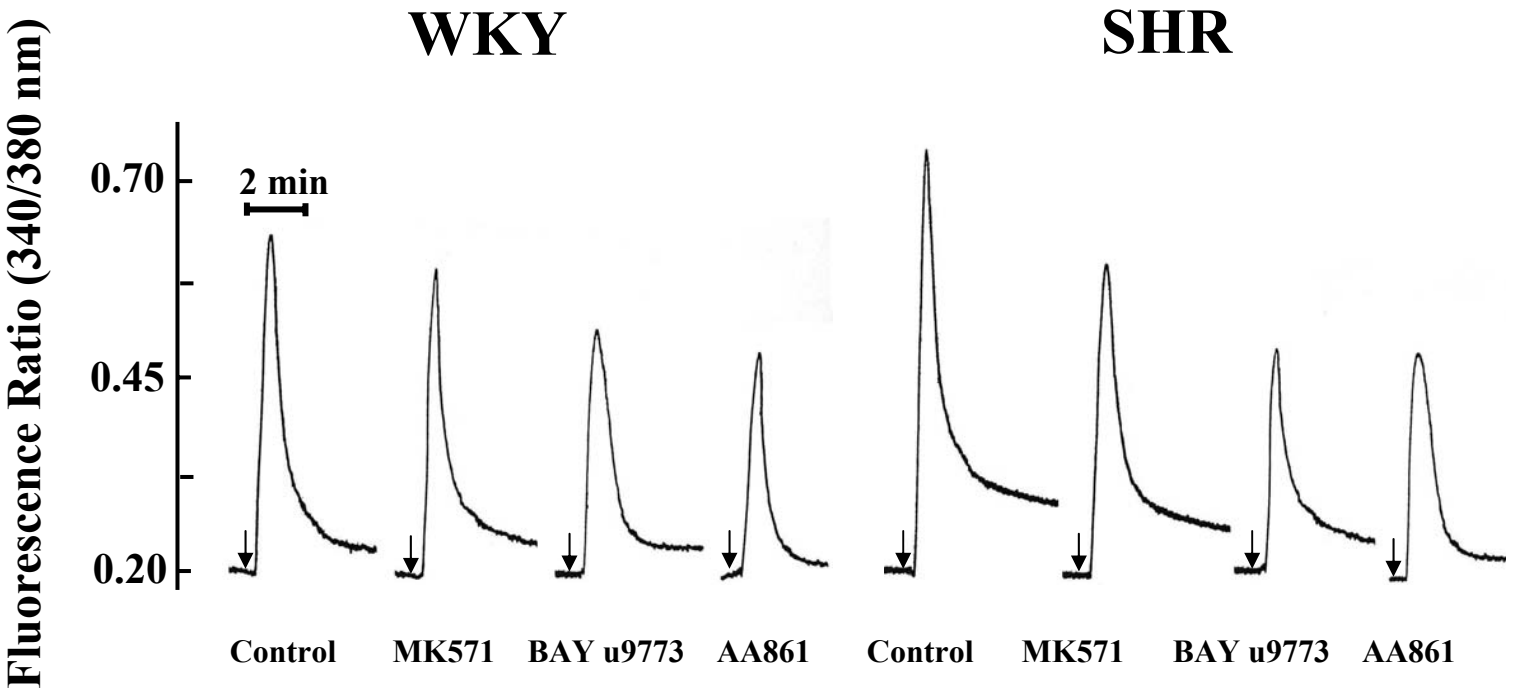


Figure 15. A representative experiment of Ang II evoked changes in the ratio of fura-2 fluoresence (340/380nm) in ASMC of WKY (left 4 tracings) and SHR (right 4 tracings) rats. ASMC loaded with fura-2 were stimulated with Ang II (100 nM) in the absent (**Control**) or in the present of either MK571 (100 nM), or Bay U9773 (100 nM) or AA861 (10 μM). These agents were added to the cuvette for 5 min. prior to the inclusion of Ang II in Krebs buffer (pH 7.4) containing normal Ca^{2+} (1.8 mM) at 37^{o}C. Note: Each sample containing fura-2 loaded ASMC was stimulated only once with a single agonist.

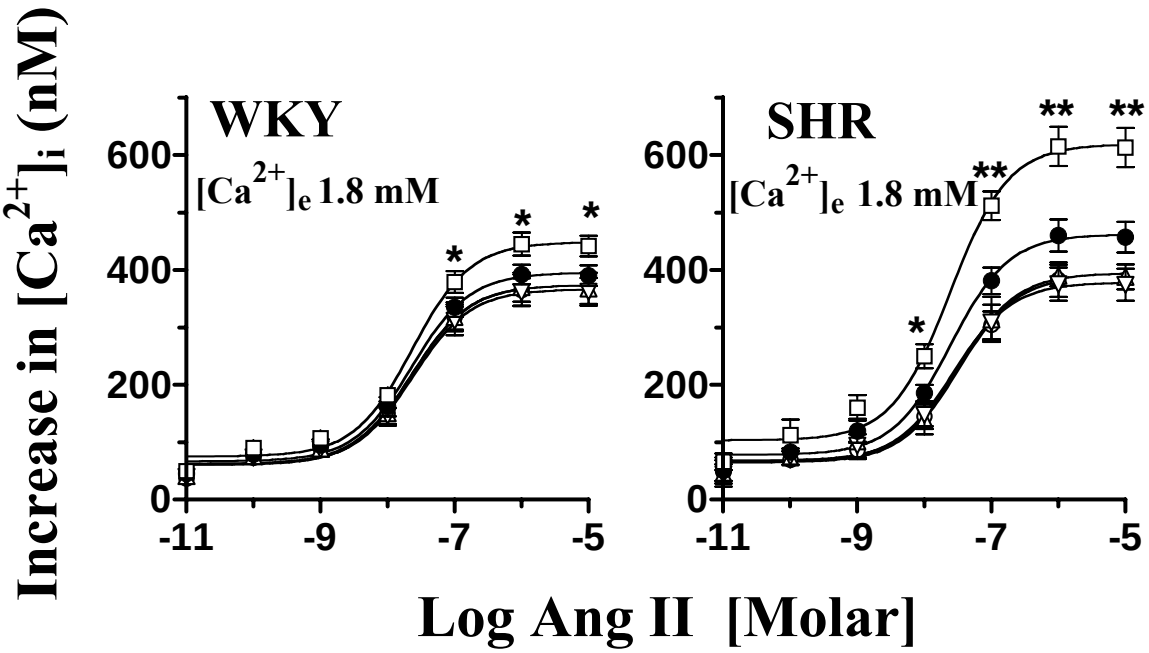


Figure 16. The effects of AA861, MK591, MK571, and Bay u9773 on peak $[Ca^{2+}]_i$ responses to Ang II in ASMC (2^{nd} –5^{th} passage) in the normal Ca^{2+} buffer. ASMC were stimulated with increasing concentrations of Ang II either in the absence (Control; □) or in the presence of AA861 (10 μM; ∇), or MK571 (100 nM; •), or MK591 (100nM; Δ) or Bay u9773 (100nM; O) in Krebs buffer (pH 7.4) at 37°C. Ang II concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being present (1.8 mM) in the buffer. Each CR curve was determined eight times using different batches of ASMC. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the MK571, AA861, MK591 and Bay u9773 treated group.

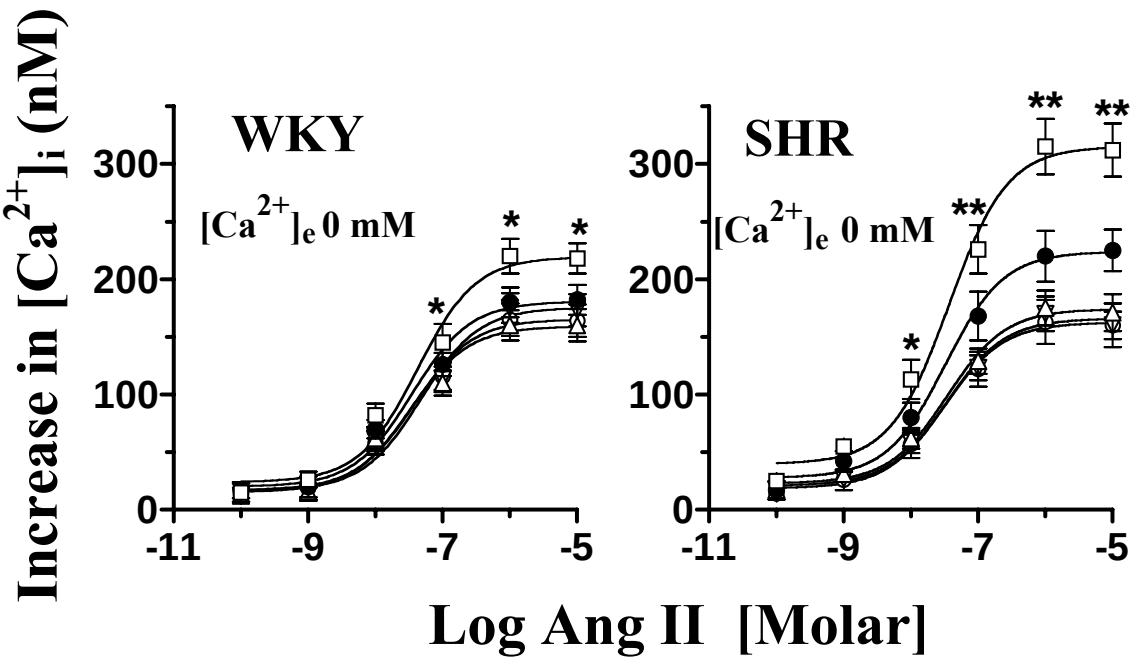


Figure 17. The effects of 5-LO inhibitor, AA861, FLAP inhibitor, MK591, $CysLT_1$ selective antagonist, MK571, and dual $CysLT_1$ / $CysLT_2$ antagonist, Bay u9773 on peak $[Ca^{2+}]_i$ responses to Ang II in ASMC (2^{nd} –5^{th} passage) in the Ca^{2+} free buffer. ASMC were stimulated with increasing concentrations of Ang II either in the absence (Control;□) or in the presence of AA861 (10 μM; ∇), or MK571 (100 nM; •), or MK591 (100nM; Δ) or Bay u9773 (100nM; O) in Krebs buffer (pH 7.4) at 37°C. Ang II concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being absent in the buffer. Each CR curve was determined eight times using different batches of ASMC. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the MK571, AA861, MK591 and Bay u9773 treated group.

4.2.4 Effects of $CysLT_1$ receptor antagonist, MK571, and dual $CysLT_1/CysLT_2$ receptor antagonist, Bay u9773 on Ang II evoked increase in $[Ca^{2+}]_i$ levels in ASMC

A representative tracing of Ang II evoked increases in fura-2 fluorescence ratio in the presence or absence of Bay u9773 (100 nM) is shown (Figure 15). In normal Ca^{2+} (1.8 mM) buffer, either MK571 (100 nM), or Bay u9773 (100 nM) significantly reduced Ang II evoked increases in peak $[Ca^{2+}]_i$ values in both WKY ($P < 0.01$) and SHR ($P < 0.01$) (Figure 16). The reductions in Ang II evoked responses (both the magnitude of responses and the % reduction in E_{max}) were more pronounced in SHR (WKY 12-18%; SHR 25-39%)(Figure 16 & Table 5). In both WKY and SHR, inhbition of responses was observed in Ca^{2+} free buffer. However, the E_{max} values for Ang II evoked responses were relatively lower and the percentage reduction in E_{max} values (compared with that obtained in normal Ca^{2+} buffer) attained by inclusion of these CysLT antagonists were relatively higher in Ca^{2+} free buffer (WKY 17-24%; SHR 34-49%) (Figure 17 & Table 5). More importantly, MK571 evoked blockade of peak $[Ca^{2+}]_i$ responses to Ang II was relatively lower compared to the responses seen in the presence of Bay u9773, in both normal and Ca^{2+} free buffer (Figure 16, 17 & Table 5).

4.2.5 Effects of AA 861, MK591, MK571 and Bay u9773 on AVP and ET-1 evoked increase in $[Ca^{2+}]_i$ levels in ASMC

Both AVP and ET-1 evoked concentration-dependent increases in $[Ca^{2+}]_i$ levels in ASMC of WKY and SHR (Figure 18 & 19). The E_{max} values for both AVP and ET-1 were higher in SHR than that in WKY. The EC_{50} and E_{max} values are summarized in Table 5. In the normal Ca^{2+} buffer, high concentration of either AA861 (30 μM), or MK591 (100 nM), or MK571 (100 nM) failed to affect the concentration-peak $[Ca^{2+}]_i$ response curves to AVP or ET-1 in ASMC of WKY and SHR (Figure 18 & 19).

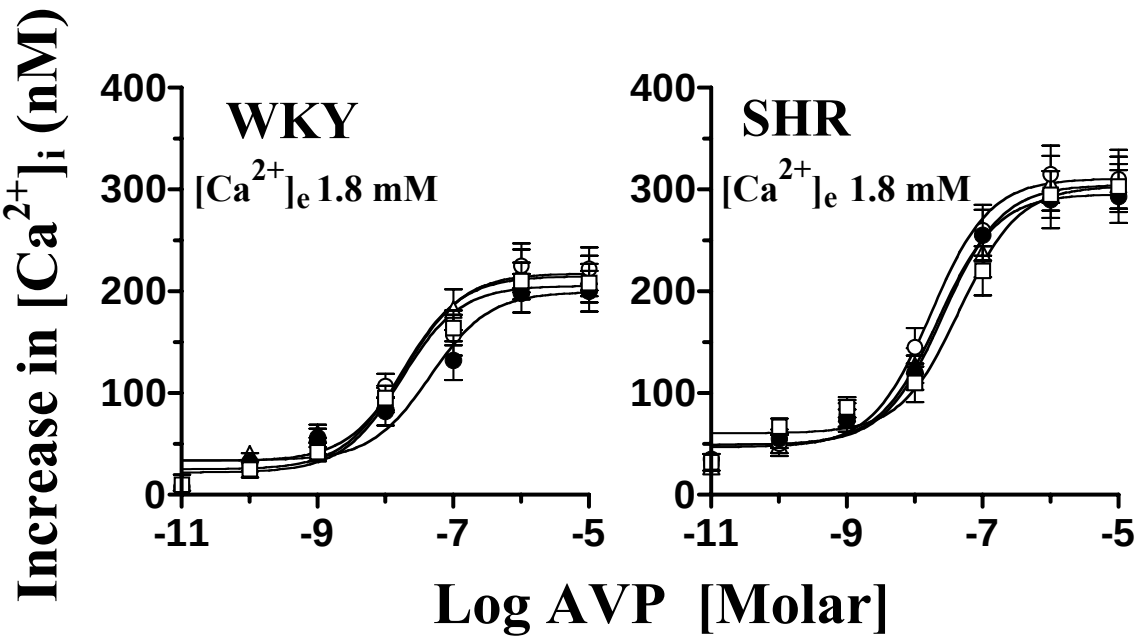


Figure 18. The effects of 5-lipoxygenase inhibitor, AA861, $CysLT_1$ selective antagonist, MK571, and dual $CysLT_1$ / $CysLT_2$ antagonist, Bay u9773 on peak $[Ca^{2+}]_i$ responses to AVP in ASMC in the normal Ca^{2+} buffer. ASMC (2^{nd} – 5^{th} passages) were stimulated with increasing concentrations of AVP either in the presence or in the absence (Control; □) of AA861 (30 μM; ●), or MK571 (100 nM; ○), or Bay u9773 (100nM; Δ) in Krebs buffer (pH 7.4) at 37°C. AVP concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} (1.8 mM) being present in the buffer. Each CR curve was determined six times using different batches of ASMC.

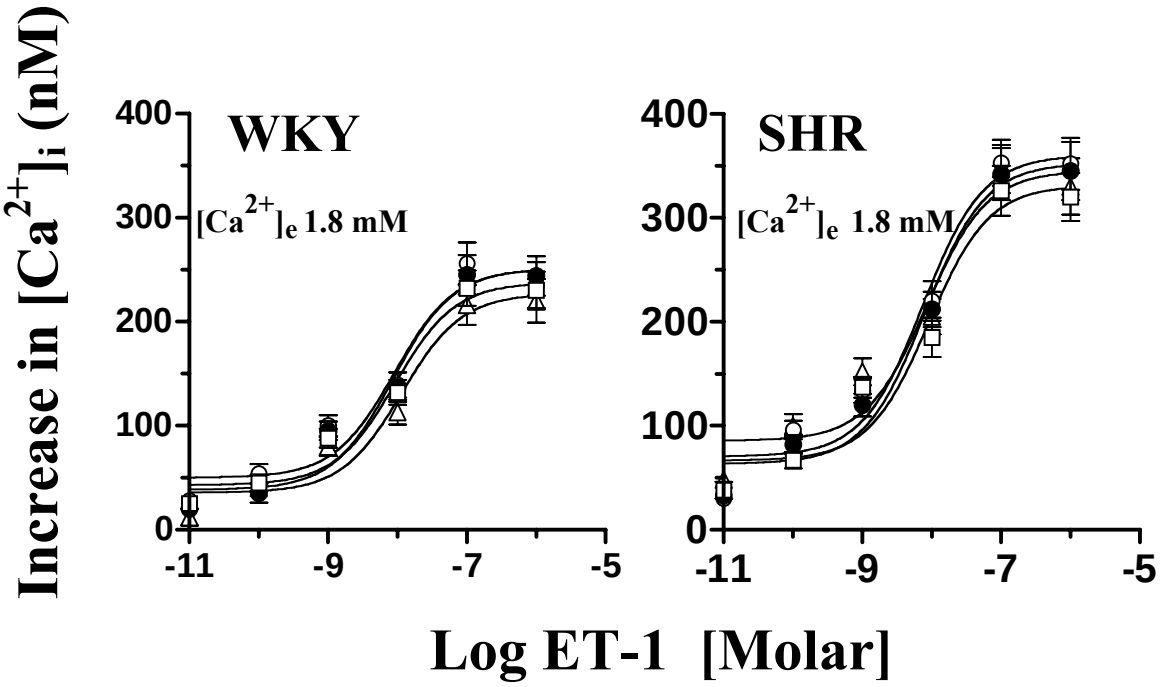


Figure 19. The effects of 5-lipoxygenase (5-LO) inhibitor, AA861, $CysLT_1$ selective antagonist, MK571, and dual $CysLT_1$ / $CysLT_2$ antagonist, Bay u9773 on peak $[Ca^{2+}]_i$ responses to ET-1 in ASMC in the normal Ca^{2+} buffer. ASMC (2^{nd} – 5^{th} passages) were stimulated with increasing concentrations of ET-1either in the presence or in the absence (Control; □) of AA861 (30 μM; ●), or MK571 (100 nM; ○), or Bay u9773 (100nM; Δ) in Krebs buffer (pH 7.4) at 37°C. ET-1 concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} (1.8 mM) being present in the buffer. Each CR curve was determined six times using different batches of ASMC.

4.2.6 Effects of AT_1 antagonist, losartan, AT_2 antagonist, PD 123319, and $InsP_3$ antagonist, 2-APB on Ang II evoked increase in $[Ca^{2+}]_i$ levels in ASMC

The $InsP_3$ blocker, 2-APB, and the AT_1 selective antagonist, losartan significantly reduced the responses to Ang II, but the AT_2 selective antagonist, PD123319, failed to block the responses to Ang II (Table 5 & Figures 20 & 21). However, both agents failed to affect the EC_{50} values for Ang II (Table 5).

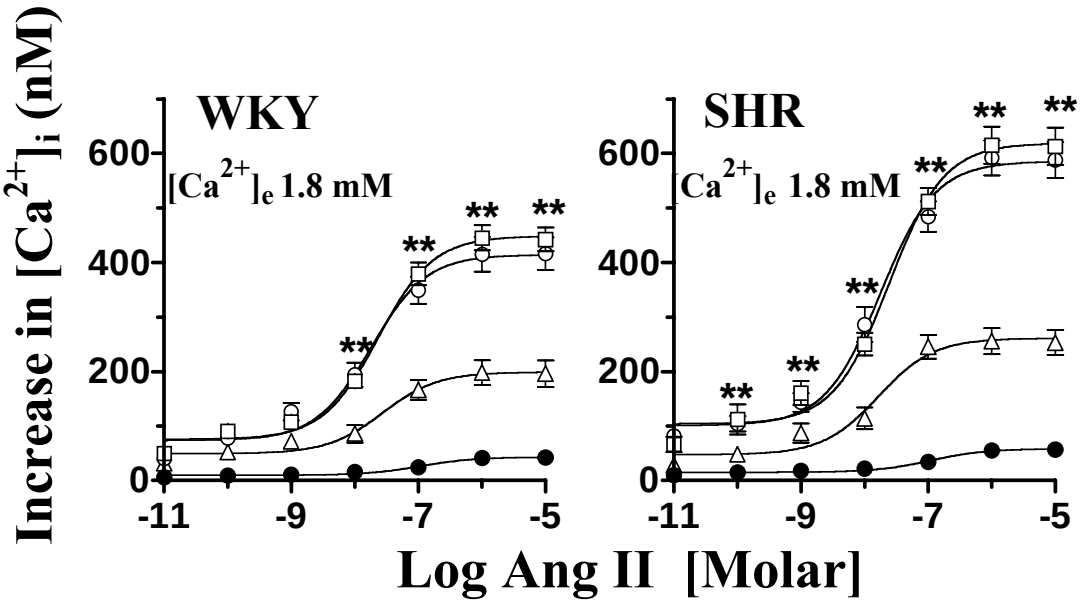


Figure 20. The effects of AT_1 receptor antagonist, losartan, AT_2 receptor antagonist, PD123319, and $InsP_3$ receptor antagonist, 2-APB on peak $[Ca^{2+}]_i$ responses to Ang II in ASMC (2^{nd} –5^{th} passage) in the normal Ca^{2+} buffer. ASMC were stimulated with increasing concentrations of Ang II either in the absence (Control; □) or in the presence of losartan (1.0 μM; ●), or PD123319 (1.0 μM; ○), or 2-APB (5 μM; Δ) in Krebs buffer (pH 7.4) at 37°C. Ang II concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being present (1.8 mM) in the buffer. Each CR curve was determined eight times using different batches of ASMC. ***P* < 0.01 compared with 2APB- and losartan-treated group.

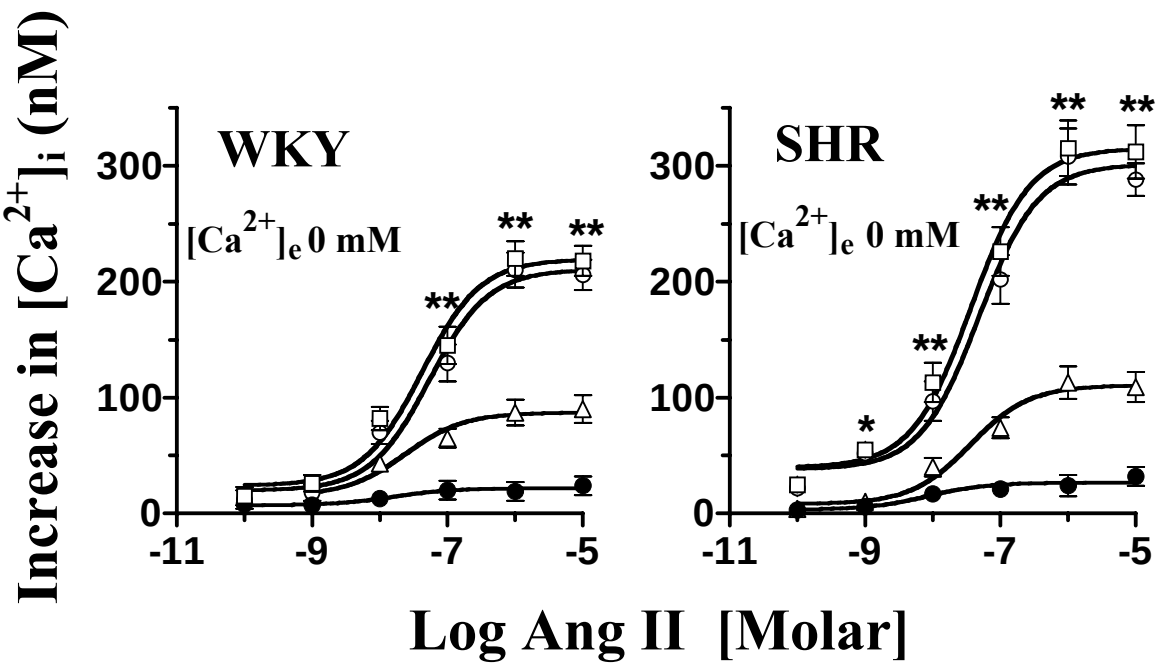


Figure 21. The effects of AT_1 receptor antagonist, losartan, AT_2 receptor antagonist, PD123319, and $InsP_3$ receptor antagonist, 2-APB on peak $[Ca^{2+}]_i$ responses to Ang II in ASMC (2^{nd} –5^{th} passage) in the Ca^{2+} free buffer. ASMC were stimulated with increasing concentrations of Ang II either in the absence (Control; □) or in the presence of losartan (1.0 μM; ●), or PD123319 (1.0 μM; ○), or 2-APB (5 μM; Δ) in Krebs buffer (pH 7.4) at 37°C. Ang II concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being absent in the buffer. Each CR curve was determined eight times using different batches of ASMC. $^{**}P < 0.01$ compared with 2APB- and losartan-treated group.

4.2.7 Both LTD_4 and LTC_4 evoked concentration-dependent increase in $[Ca^{2+}]_i$ levels in ASMC

A representative tracing of LTD_4 and LTC_4 evoked $[Ca^{2+}]_i$ response is shown (Figure 22, 23). In both normal and Ca^{2+} free buffer, LTD_4 and LTC_4 evoked increases in $[Ca^{2+}]_i$ levels with the peak responses attained in 30 to 45 seconds (Figure 22 & 23). There were no differences in the time course of the responses evoked by LTD_4 and LTC_4 in ASMC derived from either WKY or SHR strains. Both LTD_4 and LTC_4 evoked a similar degree of increases in peak $[Ca^{2+}]_i$ levels in normal Ca^{2+} and Ca^{2+} free buffer (Figure 24, 25, 26, 27). The E_{max} values for LTD_4 were higher than that for LTC_4, but much lower in comparison to the responses evoked by Ang II in the ASMC of either strain. The E_{max} values for LTD_4 and LTC_4 were higher in SHR (Figure 24, 25, 26, 27& Table 6). While Bay u9773 completely attenuated the maximal $[Ca^{2+}]_i$ responses evoked by both LTC_4 and LTD_4 in either strain, MK571 incubation led to incomplete blockade of $[Ca^{2+}]_i$ responses evoked by LTD_4 (Figure 24, 25, 26, 27& Table 6). Neither MK571 nor Bay u9773 led to significant change in the EC_{50} values for LTD_4 and LTC_4. The $InsP_3$ antagonist, 2-APB, abolished the $[Ca^{2+}]_i$ responses evoked by both LTC_4 and LTD_4 (Figure 24, 25, 26, 27& Table 6).

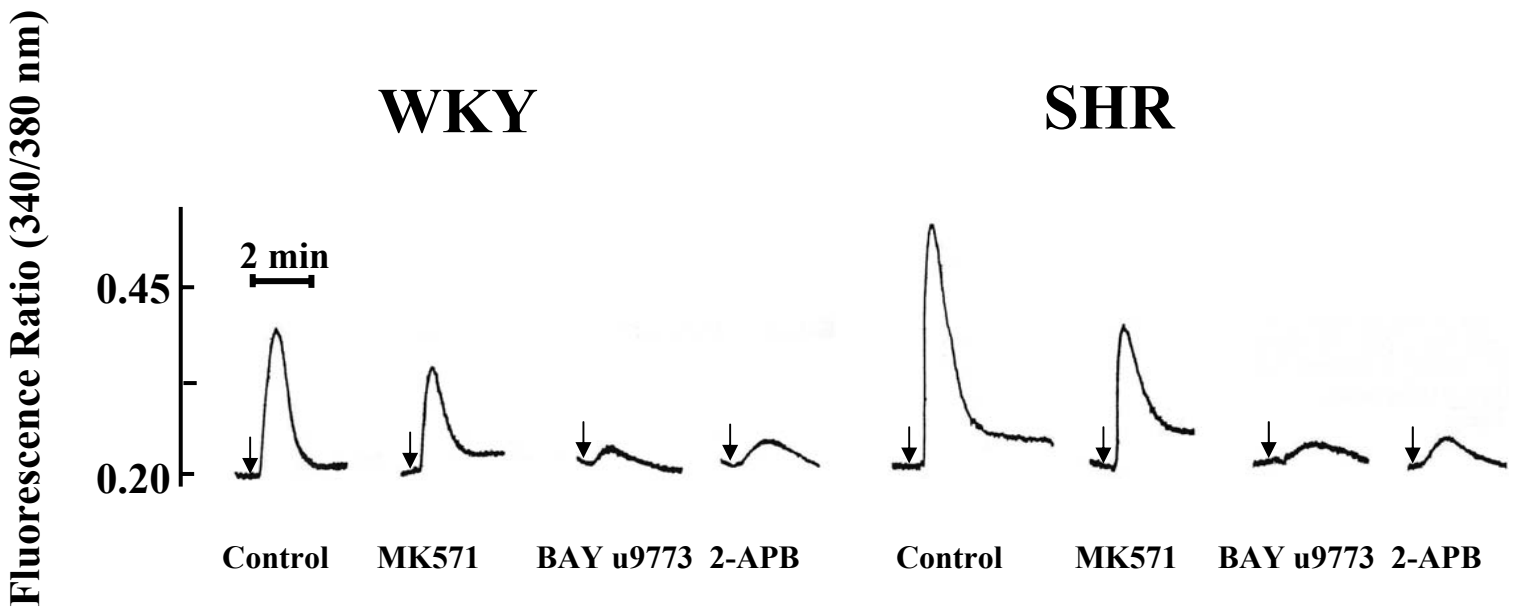


Figure 22. A representative experiment of LTD_4 evoked changes in the ratio of fura-2 fluoresence (340/380nm) in ASMC of WKY (left 4 tracings) and SHR (right 4 tracings) rats. ASMC loaded with fura-2 were stimulated with LTD_4 (1.0 μM) in the absent (**Control**) or in the present of either MK571 (100 nM), or Bay U9773 (100 nM) or 2-APB (5 μM). These agents were added to the cuvette for 5 min. prior to the inclusion of LTD_4 in Krebs buffer (pH 7.4) containing normal Ca^{2+} (1.8 mM) at 37°C. Note: Each sample containing fura-2 loaded ASMC was stimulated only once with a single agonist.

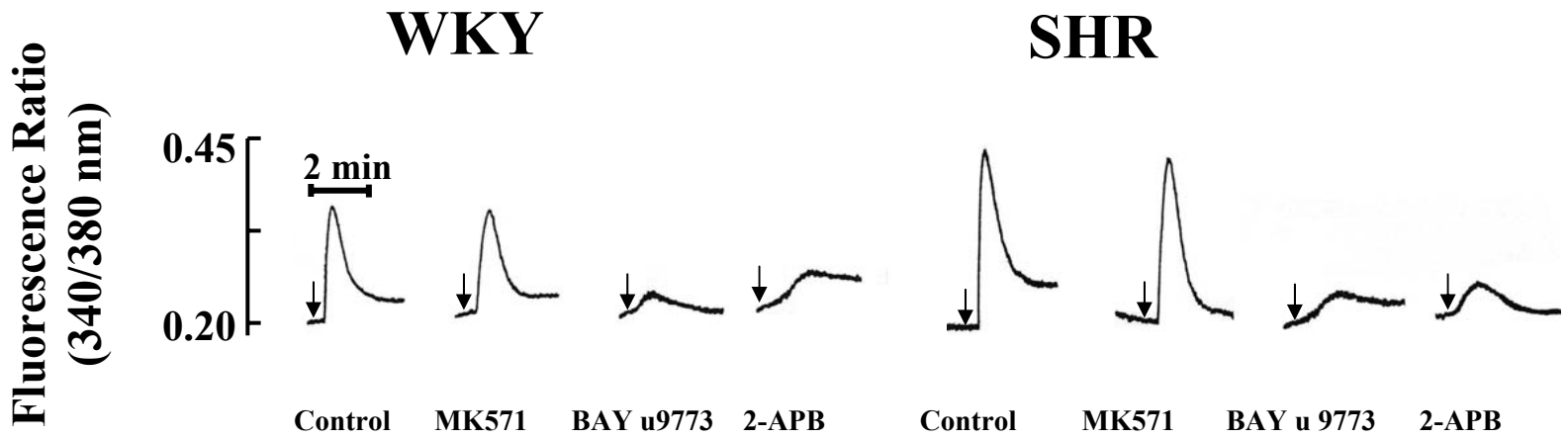


Figure 23. A representative experiment of LTC_4 evoked changes in the ratio of fura-2 fluoresence (340/380nm) in ASMC of WKY (left 4 tracings) and SHR (right 4 tracings) rats. ASMC loaded with fura-2 were stimulated with LTC_4 (1.0 μM) in the absent (**Control**) or in the present of either MK571 (100 nM), or Bay u9773(100 nM) or 2-APB (5 μM). These agents were added to the cuvette for 5 min. prior to the inclusion of LTC_4 in Krebs buffer (pH 7.4) containing normal Ca^{2+} (1.8 mM) at 37°C. Note: Each sample containing fura-2 loaded ASMC was stimulated only once with a single agonist.

Table 6. Analyses of cysteinyl leukotrienes (LTD_4 and LTC_4, 1 μM) evoked increases in $[Ca^{2+}]_i$ either in the presence or in the absence of the different inhibitors in ASMC form WKY and SHR

	WKY		SHR		WKY		SHR	
	$[Ca^{2+}]_e$ 1.8 mM				$[Ca^{2+}]_e$ 0 mM + 1.0 mM EGTA			
Agonist	EC_{50} (nM)	E_{max} (nM)	EC_{50} (nM)	E_{max} (nM)	EC_{50} (nM)	E_{max} (nM)	EC_{50} (nM)	E_{max} (nM)
LTD_4								
Control	20 ± 5	99 ± 13	21 ± 3	181 ± 17 ^^	35 ± 5	87 ± 10	19 ± 3	171± 18^^
MK571	23 ± 4	55 ± 10**	24 ± 4	91 ± 11**	28 ± 4	52 ± 8*	26 ± 4	85 ±10**
Bay u9773	ND	18 ± 6**	ND	25 ± 9**	ND	19 ± 7**	ND	21± 5**
2-APB	ND	23 ± 5**	ND	32 ± 9**	ND	27 ± 9**	ND	29 ± 7**
LTC_4								
Control	27 ± 4	70 ± 7	15 ± 3	116 ± 12^^	25 ± 4	62 ± 7	25 ± 4	109 ± 10^^
MK571	30 ± 5	58 ± 8	18 ± 3	98 ± 10	30 ± 5	54 ± 5	31 ± 5	93 ± 10
Bay u9773	ND	16 ± 6**	ND	18 ± 6 **	ND	17 ± 5**	ND	20 ± 6**
2-APB	ND	22± 6**	ND	28 ± 7**	ND	21± 7**	ND	32 ± 6**

Values shown are mean ± SEM of six experiments. The basal levels of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of ASMC in the absence of agonist stimulation were **97 ± 12** nM and **135 ± 13** nM for WKY and SHR respectively. Either AA861 (10μM), or MK591 (10 nM), or MK571(100 nM), or Bay u9773(100 nM) were added for 3 min prior to the addition of agonist. * $P<0.05$, ** $P<0.01$ compared to respective control group in the same strain rats. ** $P< 0.01$ compared to the control of WKY strain.

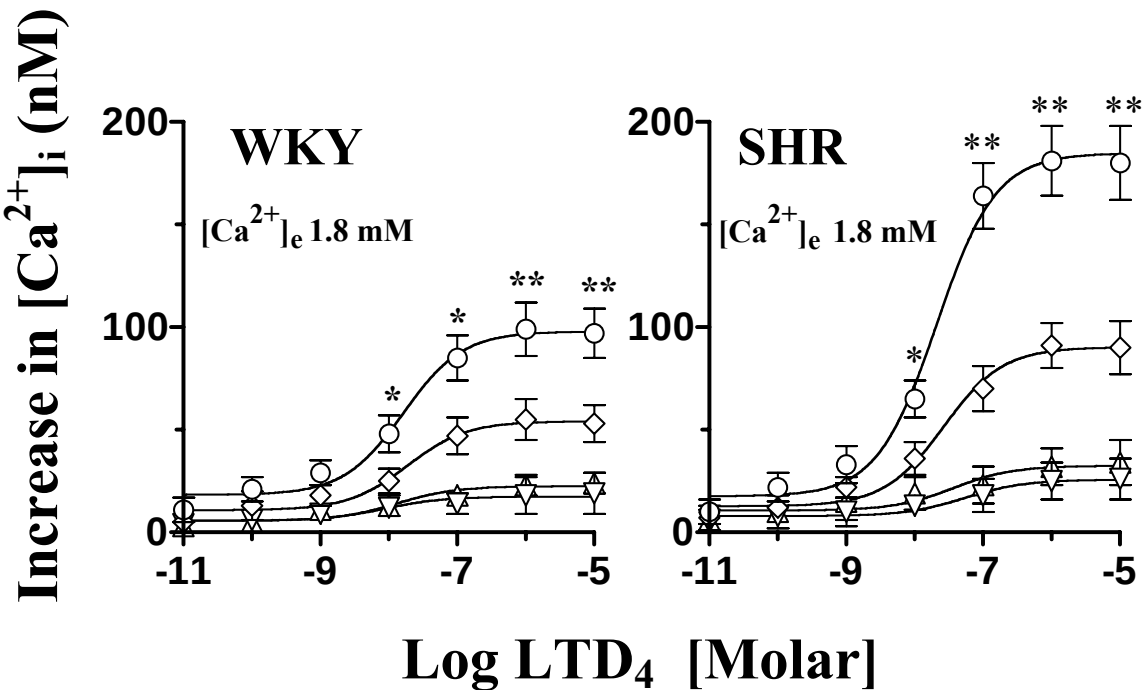


Figure 24. The effects of $CysLT_1$ selective antagonist, MK571, dual $CysLT_1$ /$CysLT_2$ antagonist, Bay u9773, and $InsP_3$ receptor antagonist, 2-APB on peak $[Ca^{2+}]_i$ responses to LTD_4 in ASMC (2^{nd} –5^{th} passage) in the normal Ca^{2+} buffer. ASMC were stimulated with increasing concentrations of LTD_4 either in the absence (Control;○) or in the presence of MK571 (100 nM; ◊), or Bay u9773 (100nM; ∇), or 2-APB (5 μM; Δ) in Krebs buffer (pH 7.4) at 37°C. LTD_4 concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being present (1.8 mM) in the buffer. Each CR curve was determined seven times using different batches of ASMC. $^{*}P< 0.05$, $^{**}P< 0.01$ compared with the MK571, Bay u9773 and 2-APB treated group.

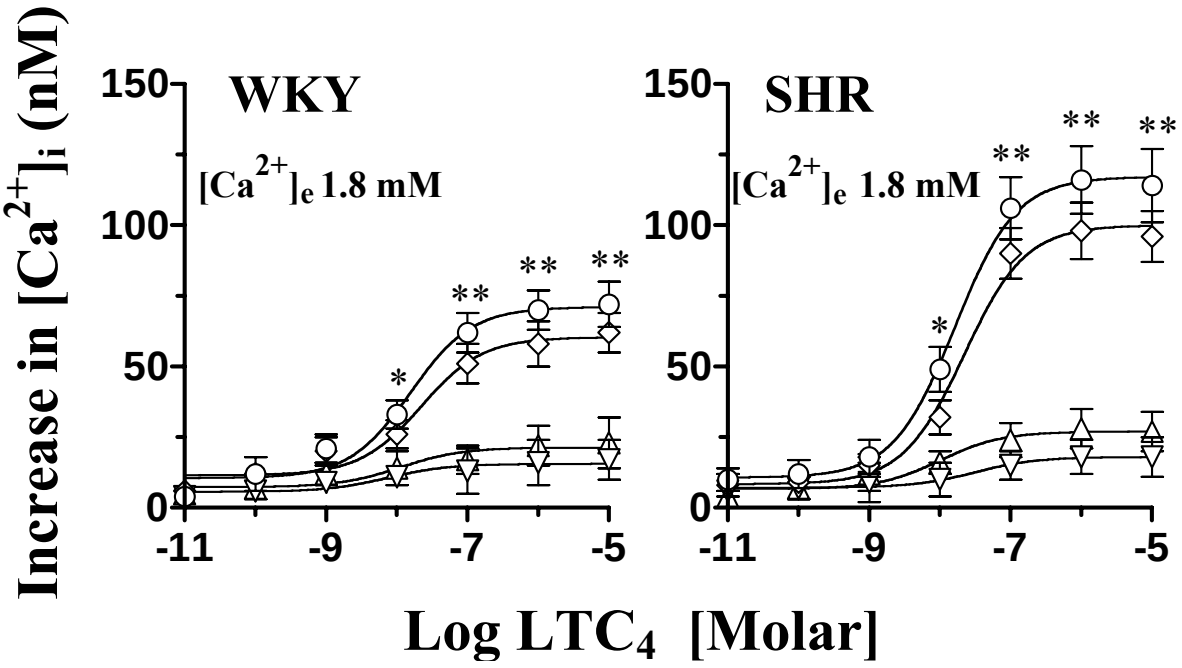


Figure 25. The effects of $CysLT_1$ selective antagonist, MK571, dual $CysLT_1/CysLT_2$ antagonist, Bay u9773, and $InsP_3$ receptor antagonist, 2-APB on peak $[Ca^{2+}]_i$ responses to LTC_4 in ASMC (2^{nd} –5^{th} passage) in the normal Ca^{2+} buffer. ASMC were stimulated with increasing concentrations of LTC_4 either in the absence (Control;O) or in the presence of MK571 (100 nM; ◊), or Bay u9773 (100nM; ∇), or 2-APB (5 μM; Δ) in Krebs buffer (pH 7.4) at 37°C. LTC_4 concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being present (1.8 mM) in the buffer. Each CR curve was determined seven times using different batches of ASMC. $^{**}P < 0.01$ compared with the MK571, Bay u9773 and 2-APB treated group.

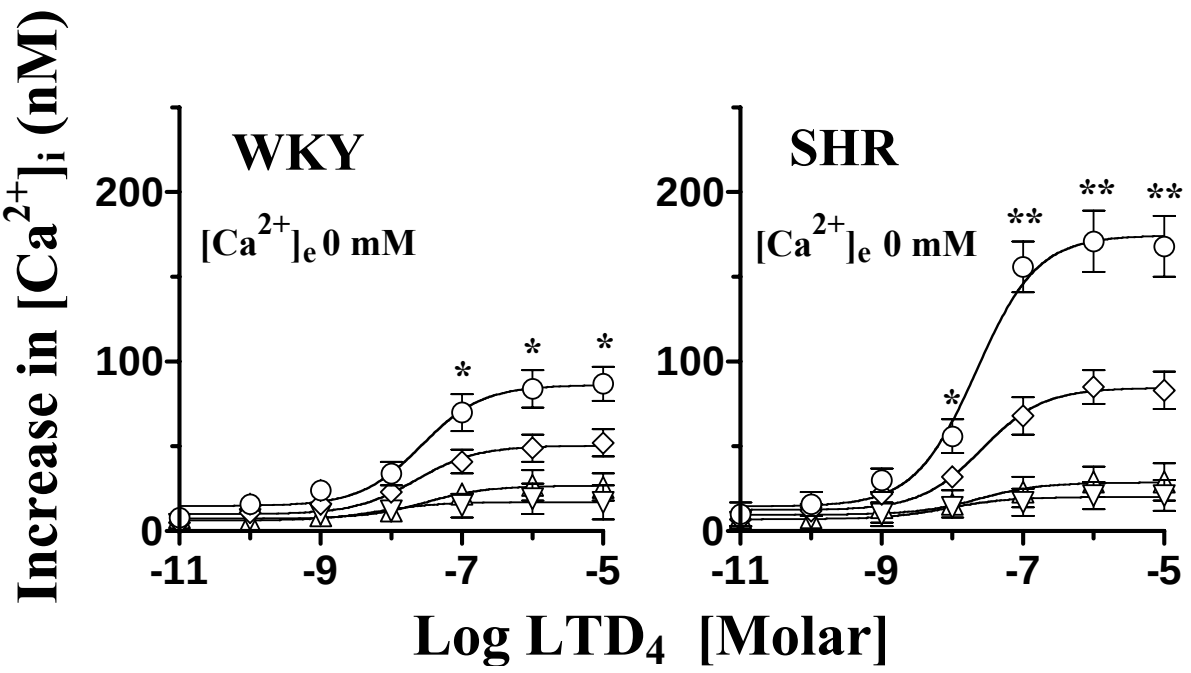


Figure 26. The effects of $CysLT_1$ selective antagonist, MK571, dual $CysLT_1/CysLT_2$ antagonist, Bay u9773, and $InsP_3$ receptor antagonist, 2-APB on peak $[Ca^{2+}]_i$ responses to LTD_4 in ASMC (2^{nd} –5^{th} passage) in the Ca^{2+} free buffer. ASMC were stimulated with increasing concentrations of LTD_4 either in the absence (Control;O) or in the presence of MK571 (100 nM; ◊), or Bay u9773 (100nM; ∇), or 2-APB (5 μM; Δ) in Krebs buffer (pH 7.4) at 37°C. LTD_4 concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being absent in the buffer. Each CR curve was determined six times using different batches of ASMC. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the MK571, Bay u9773 and 2-APB-treated groups.

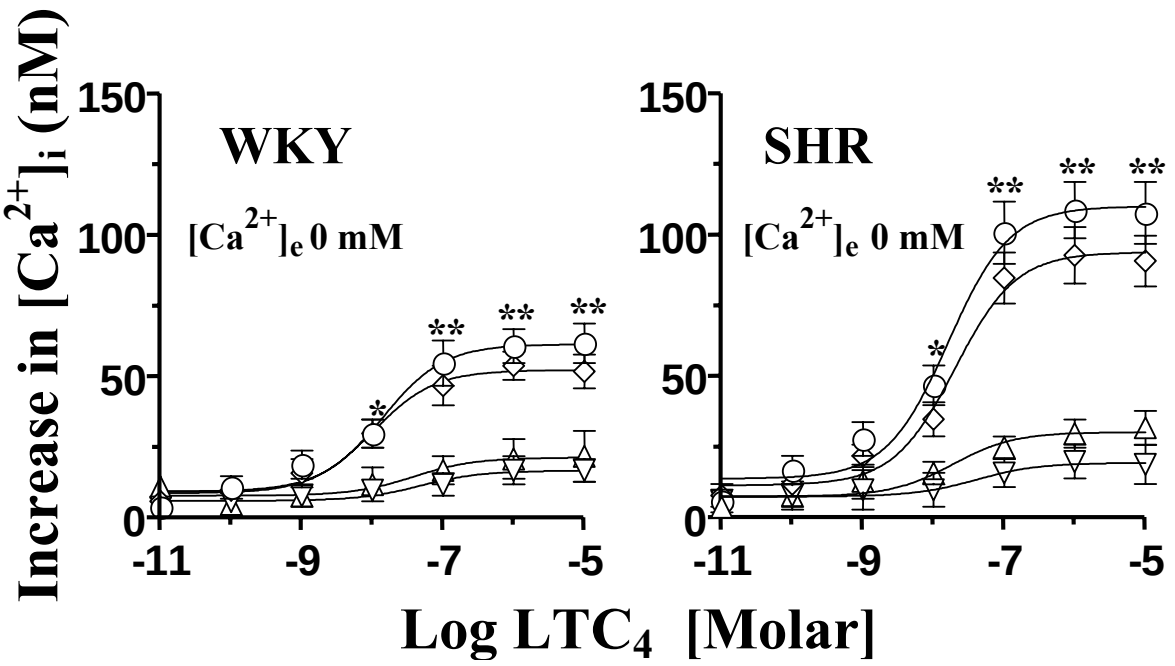


Figure 27. The effects of $CysLT_1$ selective antagonist, MK571, dual $CysLT_1/CysLT_2$ antagonist, Bay u9773, and $InsP_3$ receptor antagonist, 2-APB on peak $[Ca^{2+}]_i$ responses to LTC_4 in ASMC (2^{nd} –5^{th} passage) in the Ca^{2+} free buffer. ASMC were stimulated with increasing concentrations of LTC_4 either in the absence (Control;○) or in the presence of MK571 (100 nM; ◊), or Bay u9773 (100nM; ∇), or 2-APB (5 μM; Δ) in Krebs buffer (pH 7.4) at 37°C. LTC_4 concentration-peak $[Ca^{2+}]_i$ responses (CR) were determined with Ca^{2+} being absent in the buffer. Each CR curve was determined six times using different batches of ASMC. $^{**}P < 0.01$ compared with the Bay u9773 and 2-APB-treated groups.

4.2.8 Effects of 5-LO inhibitor, AA861, and $CysLT_1$ antagonist, MK571 on Ang II evoked increase in $[Ca^{2+}]_i$ levels in single ASMC

Single cell Ca^{2+} imaging studies revealed qualitatively similar results. A typical experiment performed with Ang II stimulation (50 nM) on adherent ASMC on four different coverslips loaded with fura-2 is shown (Figure 28). The data from single cell fluorescence determination from several experiments is summarized (Table 2). Although single cell E_{max} values for Ang II were higher when compared to data obtained with multiple cells, a similar pattern of blockade in the presence of either MK591, or AA861, or MK571, or Bay u9773 was evident. Between 2 and 3 min intervals the cells had reached steady state fluorescence close to the basal value suggesting the fluorescence changes are consistent with $[Ca^{2+}]_i$ changes and that the results gathered were not due to photobleaching (data not shown).

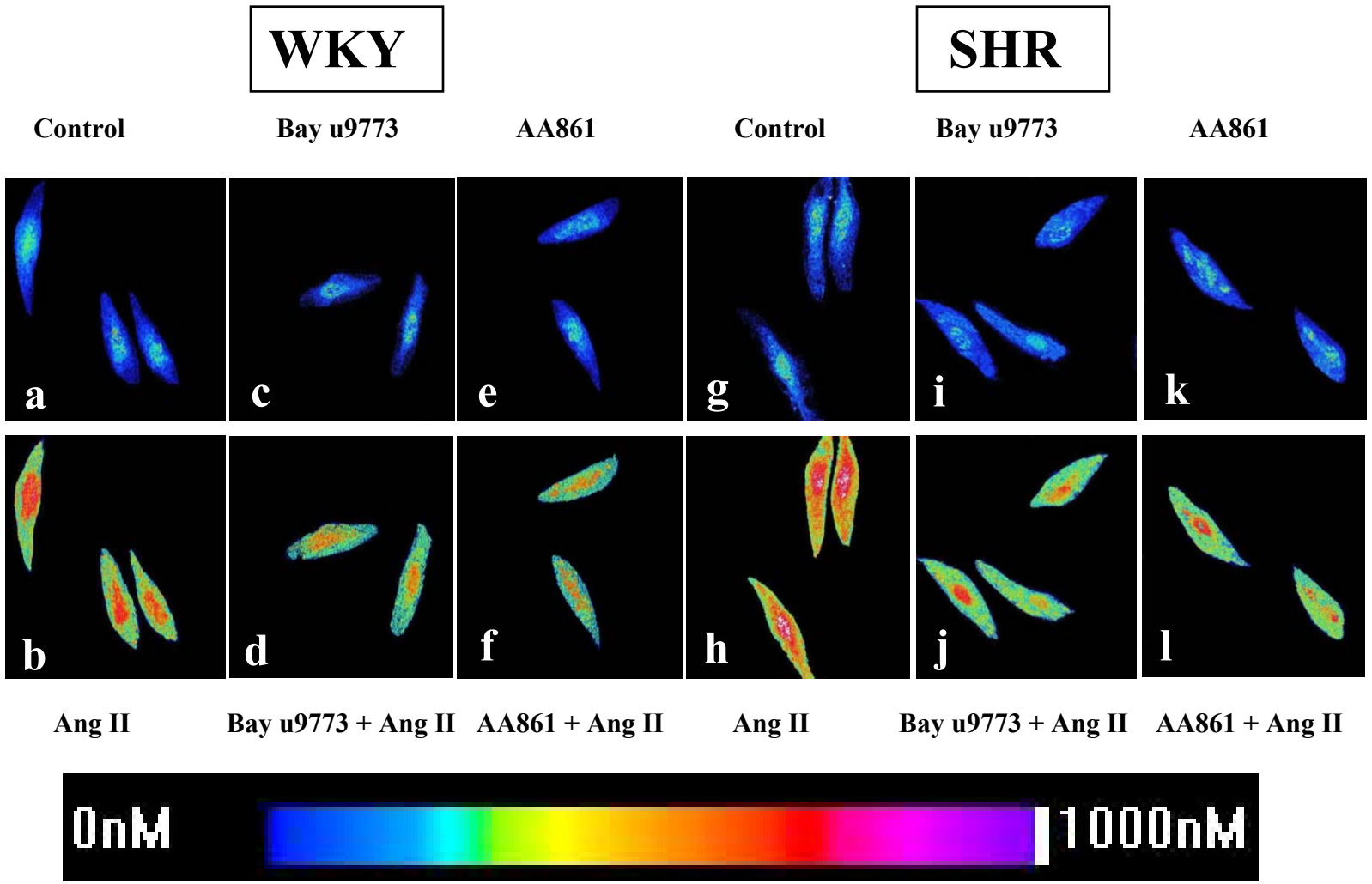

WKY
SHR
Control
Bay u9773
AA861
Control
Bay u9773
AA861
a
c
e
g
i
k
b
d
f
h
j
l
Ang II
Bay u9773 + Ang II
AA861 + Ang II
Ang II
Bay u9773 + Ang II
AA861 + Ang II
0nM
1000nM

Figure 28. Fura-2 Ca^{2+} imaging in single ASMC (**WKY: a, b, c, d, e, f; SHR: g, h, i, j, k, l**) stimulated with Ang II (50 nM). Ca^{2+} images were acquired at a rate of 3 images per second, using six different coverslips loaded with fura-2 under identical conditions on the same day. The upper panels (**a, c, e, g, i, k**) depict basal fura-2 fluorescence images (340/380 nm excitation ratio) acquired before the addition of Ang II in the absence (**a, g**) or in the presence of Bay u9773 (**c, i;**100 nM) or AA861 (**e, k;** 10 μM). The lower panels show changes in fura-2 fluorescence at 30 seconds after the addition of Ang II in the same cells (**b, h** – Ang II alone; **d, j**- Ang II in the presence of Bay u9773; **f, l** - Ang II in the presence of AA 861). At the end of two min. the fluorescence values were less than 200 nM in all the cells (data not shown). Similar response patterns were recorded in five separate experiments.

4.2.9 Effects of Ang II, AVP and ET-1 on total CysLTs production in ASMC

The basal CysLT levels (pg/10^6 cells) in the culture medium were 13.2 ± 2.7 and 23.8 ± 3.8 ($P < 0.01$) for WKY and SHR respectively. Ang II but not ET-1 neither AVP evoked a rapid, time-dependent and concentration-dependent increases in total CysLT levels in either strain (Figure 29 & 30). The E_{max} value for Ang II was much higher in SHR (26.1 ± 4.3 vs 62.6 ± 5.8, $P < 0.01$), which was observed at 30 seconds after the addition of Ang II and remained at the same level until 5 min followed by a gradually slow decrease (Figure 29).

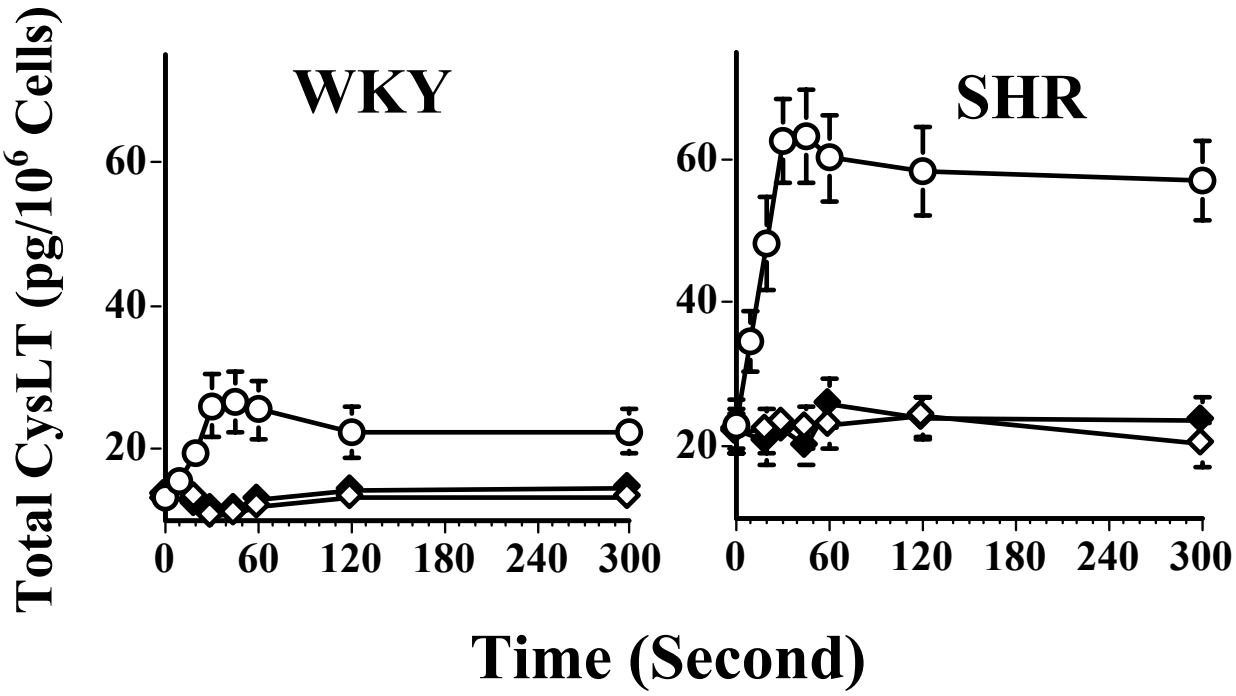


Figure 29. The time dependence of Ang II- but not ET-1- neither AVP- evoked increases in total CysLT levels in the culture medium of ASMC of WKY (Left Panel) and SHR (Right Panel). The values expressed in the graph represent the total CysLT levels in the medium at different time points following stimulation with a fixed concentration of Ang II (100 nM; ○), AVP (100 nM; Δ) and ET-1 (100 nM; □).

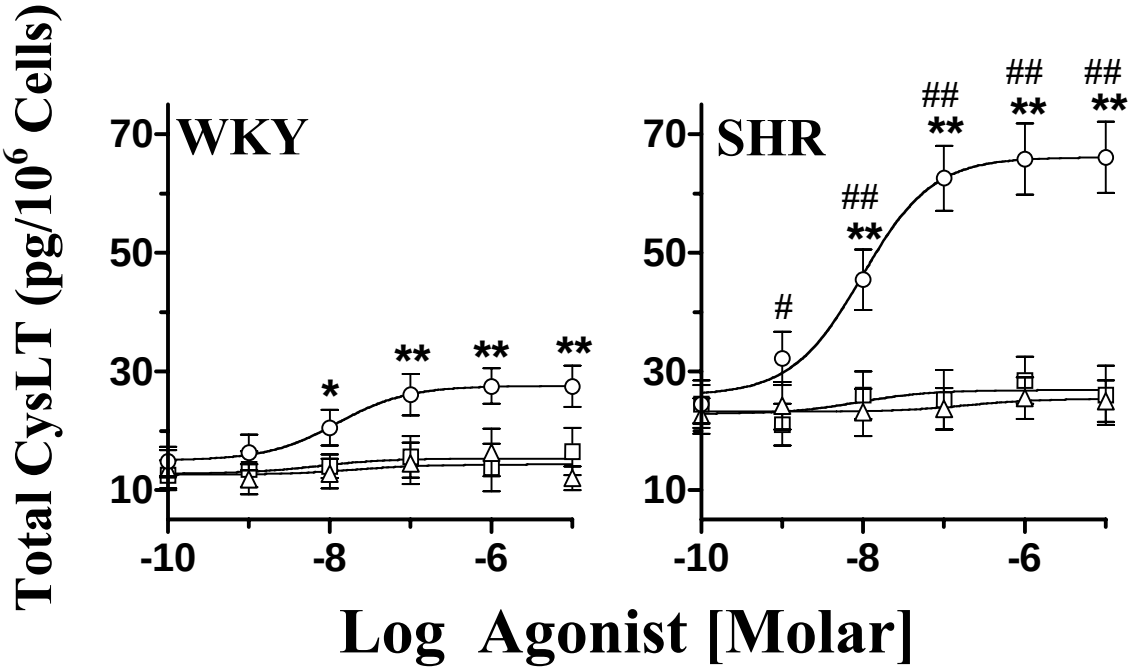


Figure 30. Ang II but not ET-1 neither AVP–evoked concentration-dependent increases in total CysLT levels in ASMC of WKY and SHR rats. The values expressed in the graph represent the total CysLT levels in the culture medium at one min. after stimulation with increasing concentrations of Ang II (○), ET-1(□) and AVP(Δ). * $P < 0.05$, ** $P < 0.01$ compared to the respective ET-1- or AVP- treated group. # $P < 0.05$, ## $P < 0.01$ compared to the respective WKY group.

4.2.10 Effects of AA861, MK591, losartan and PD123319 on Ang II evoked total CysLTs production in ASMC

Either losartan, or MK591, or AA861 but not PD123319 completely blocked Ang II-evoked total CysLT release (Figure 31). None of the inhibitors or antagonists affected the basal CysLT level. CysLT generation reached maximum at 30 seconds while the peak $[Ca^{2+}]_i$ increase was reached at 40 seconds after the addition of Ang II (100 nM) suggesting that the increased CysLT generation evoked by Ang II may contribute to elevation in $[Ca^{2+}]_i$ levels (Figure 15 & 29).

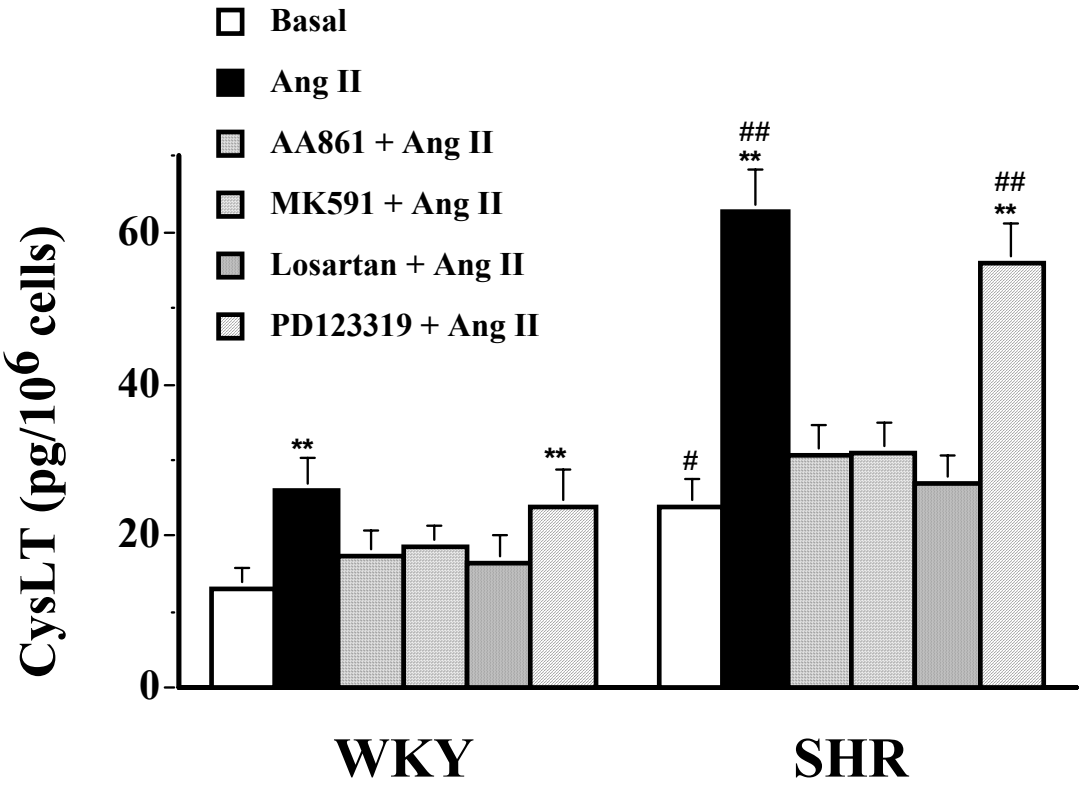


Figure 31. The effects of losartan, PD123319, AA861 and MK591 on Ang II–evoked increases in total CysLT levels in ASMC of WKY and SHR. Bar graphs provide a comparison of elevation in total CysLT levels attained in the medium one min. after stimulation with Ang II (100 nM) either in the absence (Ang II alone) or in the presence of losartan (1 μM), or PD123319 (1 μM), or AA861 (10 μM), or MK591 (100 nM). ***P* < 0.01 compared to the respective Basal level group. # *P* < 0.05, ## *P* < 0.01 compared to the respective WKY group. Basal levels represent the total CysLT levels generated by ASMC in 2 hours prior to the inclusion of any agonists, or antagonists, or inhibitors.

4.2.11 $CysLT_1$ and $CysLT_2$ receptor mRNA expression in ASMC

$CysLT_1$ and $CysLT_2$ receptor mRNAs were detected in ASMC of both strains, the expressions of both receptor were much higher in SHR ($CysLT_1$ / β actin (%): 3.9 ± 0.6 vs 16.5 ± 1.5, $p<0.01$; $CysLT_2$ / β actin (%): 3.2 ± 0.6 vs 10.9 ± 1.3, $p<0.01$) (Figure 32).

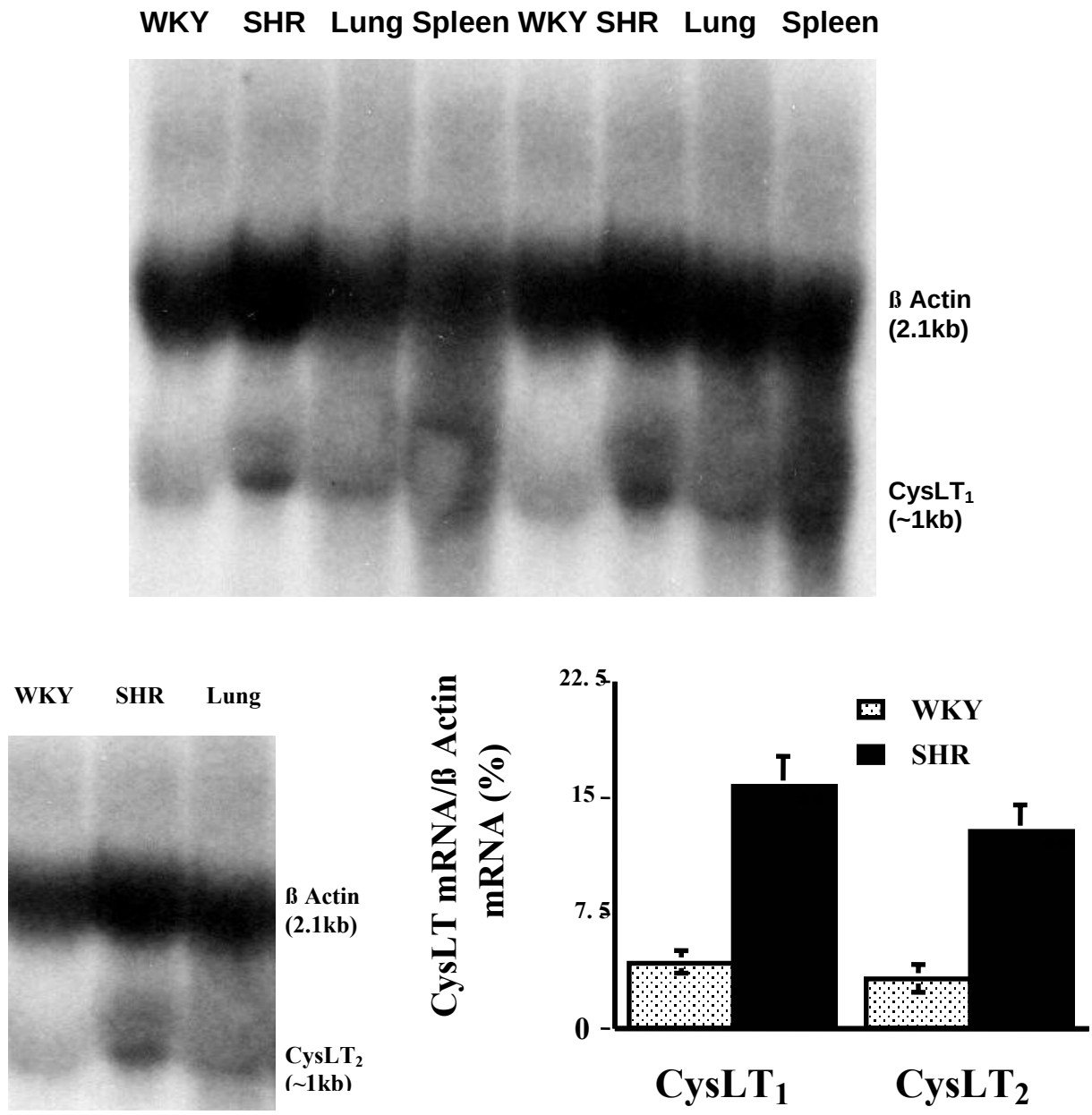


Figure 32. A representative experiment showing $CysLT_1$ and $CysLT_2$ mRNA expressed in ASMC of WKY and SHR rats. Total RNAs of lung and spleen tissues isolated from SD rats were used as positive controls. The lower right graph represents the pooled mean ± SEM values of densitometric analysis from four different blots. ** $P < 0.01$ vs WKY group.

5. DISCUSSION

5.1 CysLT contributes to Ang II evoked Ca^{2+} signaling in NRC

5.1.1. Summary

The present study provides several new observations in NRC: i) 5-LO derived metabolites of AA, CysLT, augment $[Ca^{2+}]_i$ responses to Ang II in NRC. ii) Inclusion of 5-LO inhibitor, AA861 (10 μM), or FLAP inhibitor, MK591 (100 nM) led to significant attenuation of Ang II but not ET-1 and AVP evoked increases in peak $[Ca^{2+}]_i$ levels and CysLT release into the culture medium. iii) Ang II evoked increases in $[Ca^{2+}]_i$ levels and CysLT release in NRC are mediated by AT_1 receptor activation since losartan, an AT_1 antagonist, but not the AT_2 selective antagonist, PD123319, blocked the responses to Ang II. iv) Both LTD_4 and LTC_4 evoke $[Ca^{2+}]_i$ increase in these cells. However, the responses to these mediators were much lower than those evoked by Ang II. The $CysLT_1$ selective antagonist, MK571, significantly attenuated the $[Ca^{2+}]_i$ responses to LTD_4 but failed to inhibit the responses to LTC_4 while the dual antagonist, Bay u9773, completely blocked the $[Ca^{2+}]_i$ responses to both LTD_4 and LTC_4. v) Both Ang II and CysLT evoked increases in $[Ca^{2+}]_i$ levels were markedly attenuated by 2-APB, an agent that blocks $InsP_3$ mediated Ca^{2+} release. vi) Both $CysLT_1$ and $CysLT_2$ receptor mRNA were detected in NRC. These data confirm that both Ang II and CysLT recruit predominantly

$InsP_3$ sensitive intracellular Ca^{2+} pools in NRC. Thus, the CysLT pathway may serve as an additional amplifier pathway in sustaining direct AT_1 mediated intracellular Ca^{2+} mobilization evoked by Ang II in NRC.

5.1.2 Ang II, AVP and ET-1 evoked increases in $[Ca^{2+}]_i$ in NRC

The basal levels of $[Ca^{2+}]_i$ obtained in the normal and free Ca^{2+} buffer in the present study are consistent with previous studies (Liu et al., 1999). The basal and peak increases in $[Ca^{2+}]_i$ levels were lower in the Ca^{2+} free buffer than that in the normal Ca^{2+} buffer. These data suggest that the Ca^{2+} influx contributes to the basal Ca^{2+} level and Ang II evoked peak increases in $[Ca^{2+}]_i$. The present study demonstrates that Ang II, AVP and ET-1 evoked concentration-dependent increases in $[Ca^{2+}]_i$, and that the E_{max} values for Ang II were much higher than that for AVP and ET-1. These data are in line with the earlier studies (Liu et al., 1999; Xu et al., 1991 & 1993; Touyz et al., 1997b). Several studies in the past have established that Ang II and AVP evoked elevations in $[Ca^{2+}]_i$ in NRC stemmed predominantly from the release of Ca^{2+} from the intracellular stores and that removal of extracellular Ca^{2+} led to only a partial reduction in the peak increases in $[Ca^{2+}]_i$ evoked by Ang II and AVP (Goldenberg et al. , 2001; Kem et al., 1991; Liu et al., 1999; Touyz et al., 2000). Moreover, thapsigargin, a SR Ca^{2+} pump inhibitor, abolished the $[Ca^{2+}]_i$ response to Ang II or AVP (Kem et al., 1991; Liu et al., 1999). Therefore, the

data for Ang II-evoked responses noted in either in Ca^{2+} free medium or the significant attenuation of $[Ca^{2+}]_i$ responses to Ang II determined in the presence of 2-APB are consistent with the observation reported by earlier studies.

5.1.3 AT_1 and AT_2 subtypes on NRC

The presence of two high affinity binding sites for [^{125}I] Ang II was demonstrated in membrane preparations of NRC (Rogers et al., 1986). Each of the high affinity states of the Ang II receptor has been proposed to be distinctly linked to different phospholipid second messengers. The AT_1 subtype was suggested to promote [^{3}H] inositolphosphate accumulation via activation of PLC while the AT_2 subtype was suggested to be linked to [^{3}H] AA release via activation of PLA_2. However, other studies have demonstrated that stimulation of AT_1 receptors lead to activation of PLA_2 (Freeman EJ, 1998; Pueyo ME, 1996; Bharatula M, 1998). A few studies have also shown that Ang II induced alkalinization was sensitive to blockade by AT_2 but not AT_1 selective antagonist (Kohout et al., 1995; Lokuta et al., 1994). It was also proposed that AT_1 mediated hypertrophy by Ang II could be opposed by AT_2 mediated antigrowth events in NRC (Booz et al., 1996; van Kesteren et al., 1997). In addition, another report showed that Ang II-induced apoptosis in NRC may be mediated through activation of both AT_1 and AT_2 receptors (Goldenberg et al., 2001). In contrast to these reports, detailed characterization of AT_1

and AT_2 specific binding sites and receptor expression studies using the RT-PCR strategy have revealed the presence of only a single class of AT_1 specific binding sites on NRC (Touyz et al., 1996a). Several studies by others have also established that both the increases in $[Ca^{2+}]_i$ and all phospholipid second messengers generated by Ang II in NRC are indeed mediated *solely* by the activation of AT_1 and not the AT_2 subtype (Kem et al., 1991; Sadoshima et al., 1993; Touyz et al., 1996a). Others have established that Ang II can also activate PLA_2 and PLD leading to an elevation in AA-derived metabolites (Dulin et al., 1998; Lokuta et al., 1994; Nasjletti et al., 1998; Parmentier et al., 2001, Rao et al., 1994; Touyz et al., 2000; Zafari et al., 1999). The present study demonstrates that Ang II evoked increases in CysLT and $[Ca^{2+}]_i$ levels were abolished by losartan but not PD123319, suggesting a link between the AT_1 and PLA_2 mediated AA-derived CysLT production and PLC/$InsP_3$ mediated Ca^{2+} signaling events in NRC. This result is consistent with the previous studies (Freeman et al., 1998; Pueyo et al., 1996; Bharatula et al., 1998). Therefore AT_1 receptor activation and the resultant PLC/$InsP_3$ mediated $[Ca^{2+}]_i$ responses may also be partly mediated by AA-derived CysLT production in NRC. However, more studies using PLA_2 inhibitors and their effects on Ang II evoked changes in CysLT production appear prudent in the future to resolve these issues.

Receptor binding methods were not employed in the present study as the focus of the present study was to examine the involvement of CysLT in the Ang II-mediated elevation

in peak $[Ca^{2+}]_i$ responses and to explore which Ang II receptor subtype mediated CysLT generation. Based on the data of the present study, a conclusion of the existence of only AT_1 subtype in NRC could be reached.

5.1.4 Ang II - CysLT Interaction

Ang II is known to activate both cytosolic and membrane bound PLA_2 in various tissues including cardiomyocytes that resuls in the release of AA and formation of its vasoactive metabolites (Nasjletti, 1998; Rao GN, 1994; Touyz et al., 2000; Amadou et al., 2002; Chen et al., 1998). Earlier studies have shown that Ang II-evoked vascular hypertrophy may be at least partially mediated by non-cycloxygenase derived AA metabolites (Dulin et al., 1998; Parmentier et al., 2001; Zafari et al., 1999). Recently, studies have shown that the 5-LO inhibitor, AA861, or the $CysLT_1$ antagonist, MK571 reduced the vasoconstrictor responses to Ang II suggesting that CysLT generation and subsequent $CysLT_1$ receptor activation may mediate vasoconstriction to Ang II (Shastri et al., 2001; Stanke-Labesque et al., 2001). It is well established that both Ang II and CysLT are coupled to Ca^{2+} signaling (Touyz et al., 2000; Lynch et al., 1999; Heise et al., 2000). Therefore, it was important to provide direct evidence at the level of signal transduction for a link between Ang II, AT_1 receptor activation, CysLT generation and CysLT receptor mediated alterations in $[Ca^{2+}]_i$ levels. It is well known that cell surface receptors for ET-1 are several orders of magnitude higher than the receptors for Ang II in NRC (Booz et al., 1996). Previously, we have shown that Ang II evoked much greater

increases in $[Ca^{2+}]_i$ than both AVP and ET-1 in NRC (Xu et al., 1991; Xu et al., 1993). These data suggested that additional mechanisms and signal transduction events likely account for the much greater increases in $[Ca^{2+}]_i$ responses to Ang II. The present data confirms that Ang II increased CysLT generation via AT_1 receptor activation, which in turn augments the $[Ca^{2+}]_i$ response to Ang II. In contrast, AVP and ET-1 evoked increases in $[Ca^{2+}]_i$ levels were not reduced by the inclusion of either AA861 or MK571. Moreover, both ET-1 and AVP failed to promote CysLT production, suggesting that only Ang II promotes CysLT production via AT_1 receptor activation. This is consistent with the view that Ang II is a pleiotropic agonist that recruits multiple signaling pathways to account for its potential role in the regulation of cardiovascular function (Touyz et al., 2000). Previously, others have confirmed that Ang II-evoked increases in $[Ca^{2+}]_i$ might play a more important role than protein kinase C (PKC) activation in hypertrophic responses to Ang II in NRC (Sadoshima et al., 1995a & 1995b). The present study demonstrates consistency in the time course response for the CysLT generation and the subsequent Ca^{2+} increase, suggesting that CysLT generation evoked by Ang II may serve as an additional amplifier pathway in sustaining direct AT_1 mediated Ca^{2+} mobilization in NRC. Thus, the present study provides a possible explanation for the higher E_{max} to Ang II. Furthermore, studies have already demonstrated that the increases in $[Ca^{2+}]_i$ plays a crucial role in Ang II evoked hypertrophy in NRC (Sadoshima et al., 1995 a & b).

The observation from the present study suggests that CysLT may contribute to Ang II–evoked contractility and/or hypertrophy in cardiomyocytes. More studies on the direct effects of CysLT on the DNA and protein synthesis in NRC would provide the direct evidence for the hypertropic effect of CysLT.

5.1.5 CysLT and Ca^{2+} Signaling

Although evidence that Ang II increases CysLT generation via AT_1 receptor activation was provided by the present study, it is important to demonstrate that CysLT contributes to Ca^{2+} mobilization in NRC. There are no studies suggesting the presence of CysLT receptors in NRC. In human detrusor smooth muscle cells, it was reported that elevation in $[Ca^{2+}]_i$ levels evoked by LTD_4 were almost exclusively due to mobilization from intracellular Ca^{2+} stores (Bouchelouche et al., 2001 & 2003). In other target cells, the LTD_4 evoked Ca^{2+} response was dependent on the release of Ca^{2+} from intracellular stores and enhanced Ca^{2+} influx (Ochsner et al., 1996; Pedersen et al., 1997). The present study demonstrates that both Ang II and CysLT evoked increases in $[Ca^{2+}]_i$ levels were significantly attenuated by 2-APB. In addition, both AA861 and MK571 caused a similar degree of blockade of Ang II response in normal as well as Ca^{2+} free buffer. Thus, this is the first report to characterize CysLT evoked increase in $[Ca^{2+}]_i$ that may be mainly due to the release of Ca^{2+} from $InsP_3$ sensitive intracellular SR Ca^{2+} pools in the NRC.

5.1.6 CysLT Receptors in NRC

The human $CysLT_1$ and $CysLT_2$ receptors have been cloned and characterized only in the recent years (Heise et al., 2000; Lynch et al., 1999). With the use of a variety of techniques including in situ hybridization, Northern blotting, and RT-PCR, the presence of $CysLT_1$ and/or $CysLT_2$ transcripts in cardiac tissue has been identified (Heise et al., 2000; Kamohara et al., 2001; Lynch et al., 1999; Ogasawara et al., 2002). Consistent with this observation, we have noted that both LTD_4 (MK571 sensitive) and LTC_4 (MK571 resistant) evoke concentration-dependent $[Ca^{2+}]_i$ responses in NRC. Moreover, addition of Bay u9773 completely blocked both LTD_4 and LTC_4 evoked $[Ca^{2+}]_i$ responses. More importantly, both $CysLT_1$ and $CysLT_2$ receptor mRNA was detected by northern hybridization analysis. These data suggest that NRC may possess both $CysLT_1$ and $CysLT_2$ specific binding sites that are linked to Ca^{2+} mobilization. Previously, it was reported that the affinity of LTC_4 for $CysLT_1$ receptor was roughly 10 to 350 folds lower than that of LTD_4 (Heise et al., 2000; Lynch et al., 1999). Overall, the rank order of affinities of the CysLT for the $CysLT_1R$ and $CysLT_2R$ is $LTD_4>>LTC_4>LTE_4$ and $LTD_4=LTC_4>>LTE_4$ respectively (Heise et al., 2000, Maekawa et al., 2002). Our study demonstrates that LTD_4 induced a stronger $[Ca^{2+}]_i$ response than LTC_4 and that Bay u9773 but not MK571 blocked LTC_4 induced $[Ca^{2+}]_i$ responses. It is likely that LTD_4 evoked responses are mediated by both $CysLT_1$ and $CysLT_2$ receptors while it is likely that LTC_4 interacts at $CysLT_2$ receptors on NRC that are *insensitive* to blockade by

MK571. These observations are consistent with the earlier findings that [^{3}H] LTC_4 specific binding to human lung tissues could not be displaced at concentration ranges up to 3 μM by either $CysLT_1$ antagonists (zafirlukast, and montelukast) or LTD_4 (Aharony, 1998; Heise et al., 2000; Lynch et al., 1999). Moreover, zafirlukast resistant contractile responses to LTC_4 were observed in guinea pig trachea when LTC_4 metabolism to LTD_4 was prevented (Aharony, 1998). In $CysLT_2$-expressing clones, such as CHO-7A, CHO-8B3 and PC12 cells, both LTC_4 and LTD_4 exhibited dose-dependent increases in $[Ca^{2+}]_i$ levels that were sensitive to blockade by Bay u9773 (Ogasawara et al., 2002). The peritoneal macrophages (that express both $CysLT_1$ and $CysLT_2$ receptors) responded substantially to LTD_4 (1 μM) and only slightly to 1 μM of LTC_4 (Maekawa et al., 2002). All these studies together with our present data using NRC support the notion that LTD_4 evoked $[Ca^{2+}]_i$ responses may be mediated by both $CysLT_1$ and $CysLT_2$ receptors while the weaker $[Ca^{2+}]_i$ response evoked by LTC_4 may be mediated by $CysLT_2$ receptors.

5.1.7 Effects of CysLT on NRC

Although several studies have suggested that CysLT evoke negative inotropic effects, this has been attributed to profound coronary vasoconstriction mediated by $CysLT_2$ receptors located on coronary arteries (Kamohara et al., 2001). Consistent with this, both LTD_4 and LTC_4 *at low concentrations* have been shown to exert a positive inotropic effect on the rat myocardium and that Ang II infusion has been shown to enhance LTA_4

hydrolase activity in the rat heart (Ishisaka et al., 1999; Karmazyn et al., 1990). These findings support our observation of elevated $[Ca^{2+}]_i$ levels evoked by CysLT in NRC. Althogh the effect of CysLTs on the DNA and protein synthesis was not examined in the present study, in fact, the elevation in $[Ca^{2+}]_i$ level has been suggested to play a more critical role than PKC activation towards hypertrophy evoked by Ang II in NRC (Sadoshima et al., 1993; Sadoshima et al., 1995 a & b). Taken together with our new findings, these data provide the impetus for a more detailed characterization of the interactions between Ang II, CysLT generation and CysLT receptor mediated increase in Ca^{2+} mobilization and their relative roles and contribution to cardiac hypertrophy.

5.2. CysLT Contributes to the Exaggerated Ang II Evoked Ca^{2+} Signaling in ASMC from SHR

5.2.1. Key Findings

Firstly, the present study demonstrates for the first time that either 5-LO inhibitor or $CysLT_1$/ $CysLT_2$ receptor antagonists significantly attenuated the increases in peak $[Ca^{2+}]_i$ response evoked selectively by Ang II while the responses to other peptide agonists such as ET-1 and AVP in ASMC of both WKY and SHR strains. The blockade was more pronounced in SHR, suggesting that the 5-LO-derived AA metabolites, CysLT, contribute to Ang II evoked exaggerated $[Ca^{2+}]_i$ responses in SHR. *Secondly*, LTD_4 evoked increases in $[Ca^{2+}]_i$ was much higher than the responses evoked by LTC_4. These data confirmed the presence of $CysLT_1$ and $CysLT_2$ receptors on rat ASMC. Since the

responses to LTD_4 was attenuated by Bay u9773, a dual CysLT antagonist, it was confirmed that LTD_4 evoked responses may be mediated by activation of both $CysLT_1$ and $CysLT_2$. On the other hand, the weak $[Ca^{2+}]_i$ responses evoked by LTC_4 remained unaffected in the presence of MK571 but were abolished by Bay u9773. This confirmed that LTC_4 evoked responses may be linked to $CysLT_2$ activation in ASMC in either strain. The effects evoked by both LTD_4 and LTC_4 were more pronounced in SHR. *Thirdly*, The $InSP_3$ antagonist, 2-APB, significantly reduced the $[Ca^{2+}]_i$ responses evoked by both Ang II and CysLTs, namely, LTC_4 and LTD_4. *Fourthly*, the basal CysLT level in the culture medium of ASMC of SHR was significantly higher. Ang II but not ET-1 led to an increase in CysLT generation in the ASMC of both strains. The E_{max} values for Ang II were higher in SHR. *Fifthly,* Ang II-evoked increases in both $[Ca^{2+}]_i$ level and CysLT generation in ASMC of both WKY and SHR were mediated by AT_1 and not AT_2 subtype. *Finally,* $CysLT_1$/ $CysLT_2$ receptor mRNAs were detected in ASMC of both strains and the expression levels of both subtypes were significantly higher in the SHR strain. These data suggest that elevated CysLT production along with increased levels of CysLT receptors (both $CysLT_1$ and $CysLT_2$) may account for the exaggerated $[Ca^{2+}]_i$ responses to Ang II in SHR and that this overactive pathway may contribute to exaggerated vasoconstrictor responses to Ang II in SHR due to increased mobilization of Ca^{2+} from $InsP_3$ sensitive intracellular stores.

5.2.2 Exaggerated $[Ca^{2+}]_i$ Responses to ANG II, AVP and ET-1 in ASMC of SHR

In ASMC of WKY and SHR strains, the basal $[Ca^{2+}]_i$ levels observed in the present study in both normal (1.8 mM) and Ca^{2+} free buffer medium are consistent with previous studies (Batra et al., 1993; Touyz RM, 1997b). The basal $[Ca^{2+}]_i$ as well as the peak increases in $[Ca^{2+}]_i$ evoked by agonists were lower in the Ca^{2+} free medium than the responses seen in normal Ca^{2+} medium and these data suggest that Ca^{2+} influx plays a role in Ang II-evoked responses (Batra et al., 1993; Touyz et al., 1997b). It has been proposed that the basal $[Ca^{2+}]_i$ is higher in VSMC of SHR because of increased Ca^{2+} influx through L-type voltage-dependent calcium channels (Touyz et al., 1997b). Most importantly, the present study also demonstrates that, in both WKY and SHR, the E_{max} values for Ang II were much higher than that for AVP and ET-1. This is consistent with the suggestion that Ang II recruits multiple mechanisms in evoking its response. Moreover, the E_{max} values for Ang II, AVP and ET-1 were much higher in the ASMC of SHR than that in WKY. These data are in line with the earlier studies (Batra et al., 1993; Touyz et al., 1997b). Ang II mediates its effects on $[Ca^{2+}]_i$ by $InSP_3$-induced mobilization of Ca^{2+} from sarcoplamic reticulum, inducing a rapid and transient $[Ca^{2+}]_i$ response initially, followed by influx through Ca^{2+} channels, resulting in a prolonged and sustained $[Ca^{2+}]_i$ response (Dostal et al., 1990). Other studies showed that Ang II can

also cause calcium release from intracellular stores without involving the $InsP_3$ receptor by a calcium-induced calcium-release mechanism (Morel et al., 1996). Although the activity of calcium channels is increased in hypertension, it has been proposed that an increase in Ca^{2+} influx is not a major mechanism that contributes to Ang II-evoked exaggerated $[Ca^{2+}]_i$ responses in VSMC from SHR (Touyz et al., 1997b). Instead, it is suggested that Ang II-induced exaggerated $[Ca^{2+}]_i$ response is dependent primarily on intracellular rather than extracellular mobilization of calcium. However, the exact mechanism underlying Ang II evoked exaggerated $[Ca^{2+}]_i$ responses is still not fully understood.

5.2.3 AT_1 and AT_2 subtypes on ASMC

Both AT_1 and AT_2 receptor subtypes were expressed in VSMCs from WKY and SHR (El Mabrouk et al., 2001). In the vasculature, AT_1 receptors have been shown to mediate most of the physiological actions of Ang II, and this subtype is predominant in the control of Ang II-induced vascular functions (Sadoshima et al., 1998; Touyz et al., 2000). AT_1 receptors are present at high levels in VSMC (Touyz et al., 2000; Allen et al., 2000). AT_1 receptor has been linked to vascular remodeling because of its implication in vascular hypertrophy and/or hyperplasia, extracellular matrix deposition, and inflammatory responses (Matsusaka et al., 1997; Touyz et al., 2001). In contrast, AT_2 receptor is expressed at very high levels in the fetus and its expression is very low in

adult VSMC (Nahmias et al., 1995; Touyz et al., 2001). The physiological function of AT_2 receptors has not been clearly defined, although several studies suggest that they have a proapoptotic effect on VSMC in normal rats (Cui et al., 2001; Suzuki et al., 2003; Kumar et al., 2004). Other studies suggest that AT_2 receptors play a role in differentiation and inflammation (Ruiz-Ortega et al., 2000; Yamada et al., 1999). To date, studies have revealed controversial results concerning the role of AT_2 receptor on VSMC growth in different rat models of experimental hypertension (Levy et al., 1996; Sabri et al., 1997; Cao et al., 1999; Otsuka et al., 1998). The exact role of AT_2 receptors in the regulation of VSMC growth and in hypertension awaits further clarification. The present study demonstrates that Ang II evoked increases in CysLT and $[Ca^{2+}]_i$ levels were abolished by losartan but not by PD123319. Therefore, AT_1 receptor activation and the resultant PLC/$InsP_3$ mediated $[Ca^{2+}]_i$ responses may be partly mediated by AA-derived CysLT production in ASMC. These results support the view that AT_1 receptor activation accounts for most or all of the vascular actions of Ang II.

5.2.4 Cys LT Dependent $[Ca^{2+}]_i$ Responses to ANG II in ASMC

Studies have demonstrated that Ang II-evoked vascular hypertrophy may be mediated by noncycloxygenase-derived AA metabolites (Dulin et al., 1998, Parmentier et al., 2001,

Zafari et al., 1999). Recently, studies from our laboratories and by others have shown that 5-LO inhibitor or the $CysLT_1$ antagonist reduced the vasoconstrictor responses to Ang II, suggesting that CysLT generation and subsequent $CysLT_1$ receptor activation mediate vascular responses to Ang II (Shastri et al., 2001; Stanke-Labesque et al., 2001). Our study demonstrates that AT_1 mediated CysLT generation and the subsequent CysLT evoked Ca^{2+} release from $InsP_3$ sensitive intracellular stores could enhance direct Ang II evoked responses (Liu et al., 2003). Therefore, it is important to find direct evidence for a link between Ang II, AT_1 receptor activation, CysLT generation and CysLT receptor mediated alterations in $[Ca^{2+}]_i$ levels in ASMC. We have previously shown that Ang II evoked much greater increases in $[Ca^{2+}]_i$ than either AVP or ET-1 in ASMC (Gopalakrishnan et al.,1991), though cell surface receptors for ET-1 are several orders of magnitude higher than the receptors for Ang II (Booz et al., 1996). These data suggested that additional mechanisms and signal transduction events likely account for the much greater increases in $[Ca^{2+}]_i$ responses to Ang II. The present data confirms that Ang II increased CysLT generation via AT_1 receptor activation. In contrast, neither 5-LO inhibitors nor $CysLT_1$/ $CysLT_2$ antagonists attenuated ET-1 evoked increases in $[Ca^{2+}]_i$ levels and ET-1 failed to elicit CysLT generation, suggesting that only Ang II promotes CysLT production via AT_1 receptor activation. The present data shows that CysLT plays a more pronounced role in the exaggerated $[Ca^{2+}]_i$ response to Ang II in SHR. In

addition, the present study demonstrates the consistency in time course response for the CysLT generation and the subsequent Ca^{2+} increase, suggesting that CysLT generation evoked by Ang II may serve as an additional amplifier pathway in sustaining direct AT_1 mediated Ca^{2+} mobilization in ASMC. This is consistent with the view that Ang II is a pleiotropic peptide that recruits multiple signaling pathways to account for its potential role in the regulation of cardiovascular function (Touyz et al., 2000). Thus, besides providing a possible explanation for the higher E_{max} to Ang II, the present study suggests for the first time that CysLT may play a more important role in Ang II–evoked exaggerated vasoconstriction/or vascular hypertrophy in SHR.

5.2.5 CysLT and Ca^{2+} Signaling.

Studies have shown that CysLT signal through elevation of intracellular calcium by activation of $CysLT_1$/ $CysLT_2$ receptors (Lynch et al., 1999; Takasaki et al., 2000; Chan et al., 1994). In human detrusor smooth muscle cells, it was reported that elevation in $[Ca^{2+}]_i$ levels evoked by LTD_4 were almost exclusively due to mobilization from intracellular Ca^{2+} stores (Bouchelouche et al., 2001). In other target cells, the LTD_4 evoked Ca^{2+} response was dependent on the release of Ca^{2+} from intracellular stores and enhanced Ca^{2+} influx *(*Ochsner et al., 1996, Pedersen et al., 1997*)*. The present study demonstrates that CysLT evoked increase in $[Ca^{2+}]_i$ may be mainly due to the release of

Ca^{2+} from $InsP_3$ sensitive intracellular SR Ca^{2+} pools in the ASMC. This is consistent with the data from other studies (Mazzetti et al., 2003; Bouchelouche et al, 2000).

5.2.6 ANG II evoked CysLT generation in ASMC

The present study has demonstrated for the first time that the basal CysLT level is higher in the ASMC of SHR and Ang II evoked more pronounced increase in CysLT generation in SHR. This is the first report to provide the direct evidence for a link between Ang II and CysLT in ASMC. Other studies have reported that Ang II elicits CysLT release from the aortic rings but they failed to monitor CysLT production in the ASMC (Stanke-Labesque et al., 2001). Besides higher basal CysLT level in SHR, the present data also show that both $CysLT_1$ and $CysLT_2$ receptor mRNA were detected in ASMC of both strains and the expression levels for both receptors were much higher in SHR. This seems to go against the rule that higher level of hormones usually lead to a down-regulation of their receptors via a physiological negative feedback mechanism. However, studies dealing with Ang II, ET-1 and AVP have reported up-regulation of their receptors irrespective of their elevated levels in cardiovascular disease states (Cheng et al., 1995; Peng et al., 2002; Okamura et al., 1999; Shibataet al., 1997, Wagner et al., 1998; Tonnessen et al., 1997; Kobayashi et al., 1999). More particularly, it has been shown that Ang II upregulates AT_1 receptors in renal proximal tubules (Cheng et al., 1995). Other studies have demonstrated that both Ang II and AT_1 receptor levels are simultaneously elevated (Peng et al., 2002; Okamura et al., 1999; Shibata et al. 1997, Wagner et al., 1998). Similarly, it has been reported that the ET-1 peptide level, the ET_A- and ET_B-

receptor expression are upregulated in the heart of rats with chronic heart failure (Kobayashi et al., 1999; Tonnessen et al., 1997). All these studies support our present observation. The reason for the higher basal CysLT level and receptor expression could be attributed to the higher plasma Ang II level and overactive AT_1 activation that may enhance both 5-LO-mediated CysLT generation of CysLT-R expression in SHR. On the other hand, it is also possible that the up-regulated CysLT system serves as an additional amplifier pathway for the exaggerated $[Ca^{2+}]_i$ responses to Ang II and finally leads to the generation of hypertension and vascular hypertrophy in SHR.

5.2.7 Cys LT Receptors in ASMC

Human $CysLT_1$ and $CysLT_2$ receptors have been cloned and characterized only in recent years (Heise et al., 2000; Lynch et al., 1999). Several recent studies have proposed the presence of $CysLT_1$ and $CysLT_2$ transcripts in cardiac and vascular tissues (Heise et al., 2000; Kamohara et al., 2001; Lynch et al., 1999; Ogasawara et al., 2002). The present data have demonstrated for the first time that both $CysLT_1$ and $CysLT_2$ receptor are expressed in ASMC. Consistent with this observation, we have further noted that both LTD_4 (MK571 sensitive) and LTC_4 (MK571 resistant) evoke concentration-dependent $[Ca^{2+}]_i$ responses in ASMC. Moreover, Bay u9773 completely blocked both LTD_4 and LTC_4 evoked $[Ca^{2+}]_i$ responses, confirming that ASMC possess both $CysLT_1$ and $CysLT_2$ specific binding sites that are linked to Ca^{2+} mobilization. Previously, it was

reported that the affinity of LTC_4 for $CysLT_1$ receptor was roughly 10 to 350 fold lower than that of LTD_4 (Heise et al., 2000; Lynch et al., 1999). Overall, the rank order of affinities of the CysLT for the $CysLT_1R$ and $CysLT_2R$ is $LTD_4>>LTC_4>LTE_4$ and $LTD_4=LTC_4>>LTE_4$ respectively (Heise et al., 2000, Maekawa et al., 2002). In the present study, LTD_4 evoked stronger response than LTC_4 did in either WKY or SHR, further suggesting that LTD_4 exert its effects via activation of both $CysLT_1$ receptor and $CysLT_2$ receptor while LTC_4 mediate its effects predominantly via activation of $CysLT_2$ receptor. These results are in line with the earlier findings (Aharony, 1998; Heise et al., 2000; Lynch et al., 1999; Maekawa et al., 2002; Ogasawara et al., 2002).

In conclusion, the present study confirms that elevated CysLT production along with increased levels of CysLT receptors (both $CysLT_1$ and $CysLT_2$) may account in for part the exaggerated $[Ca^{2+}]_i$ responses to Ang II in SHR via mobilization of Ca^{2+} from $InsP_3$ sensitive intracellular stores. As it has been suggested that the elevation in $[Ca^{2+}]_i$ level play a more critical role than PKC activation towards hypertrophy evoked by Ang II (Sadoshima et al., 1993,1995 a & b), taken together with our new findings, these data provide the impetus for a more detailed characterization of the interactions between Ang II, CysLT generation and CysLT receptor mediated increase in Ca^{2+} mobilization and their relative roles and contribution to vascular hypertrophy and hypertension.

6. SUMMARY OF FINDINGS AND CONCLUSIONS

6.1 For Neonatal Rat Cardiomyocyte

1. Ang II, AVP and ET-1 evoked concentration-dependent increases in $[Ca^{2+}]_i$ levels in NRC. The E_{max} values for Ang II were much higher than that for AVP and ET-1.

2. Ang II evoked time- and concentration-dependent increases in CysLT production in NRC. Ang II evoked increases in $[Ca^{2+}]_i$ levels and CysLT production were mediated by AT_1 but not AT_2 receptors.

3. Inclusion of the 5-LO inhibitor or the FLAP inhibitor, or CysLT receptor antagonists, led to significant reduction in Ang II but not AVP or ET-1 evoked increases in $[Ca^{2+}]_i$ levels. Therefore, CysLTs may partially contribute to Ang II-evoked increase in $[Ca^{2+}]_i$ in NRC

4. Both $CysLT_1$ and $CysLT_2$ receptor mRNA were detected in NRC.

5. Both LTD_4 and LTC_4 evoke concentration-dependent increases in $[Ca^{2+}]_i$ in NRC. Moreover, the $CysLT_1$ selective antagonist, MK571, blocked LTD_4 but not LTC_4 evoked increases in $[Ca^{2+}]_i$ levels while the dual $CysLT_1$ / $CysLT_2$ receptor antagonist, Bay u9773, abolished the $[Ca^{2+}]_i$ responses to both LTD_4 and LTC_4, suggesting that LTD_4 evoked responses are mediated by both $CysLT_1$ and $CysLT_2$ receptors, while LTC_4 predominantly activate $CysLT_2$ receptors present on NRC.

6. Both Ang II and CysLTs evoked increases in $[Ca^{2+}]_i$ were markedly attenuated by 2-APB suggesting that CysLTs recruit predominantly $InsP_3$ sensitive intracellular Ca^{2+} pools in NRC. Therefore, the CysLT pathway may serve as an additional amplifier pathway in sustaining direct AT_1 mediated Ca^{2+} signaling evoked by Ang II in NRC.

6.2 For VSMC

1. Ang II, AVP and ET-1 evoked concentration increases in $[Ca^{2+}]_i$ levels in ASMC from both WKY and SHR. The E_{max} values for all these peptides were higher in SHR than that in WKY. Most importantly, The E_{max} values for Ang II were much higher than that for AVP and ET-1.

2. In both WKY and SHR, either AA861, or MK591, or MK571, or Bay u9773 led to significant reduction in Ang II but neither AVP nor ET-1 evoked increases in $[Ca^{2+}]_i$ in both normal and Ca^{2+} free buffer. Also, AT_1 but not AT_2 receptor antagonists completely blocked Ang II-evoked increase in $[Ca^{2+}]_i$ levels.

3. The basal CysLT level is much higher in SHR than that in WKY. Ang II but not AVP or ET-1 evoked time- and concentration- dependent increases in CysLT generation in the culture medium of ASMC from both WKY and SHR. The E_{max} values for Ang II were much higher in SHR than that in WKY. AT_1 but not AT_2 receptor antagonist completely

blocked Ang II-evoked increase in CysLT level. Both 5-LO inhibitor and FLAP inhibitor also abolished Ang II-evoked response. The blockade effects were more profound in SHR, suggesting that CysLTs may play a role in Ang II-evoked exaggerated vasoconstrictor responses seen in SHR strain.

4. LTD_4 and LTC_4 evoked concentration-dependent increases in $[Ca^{2+}]_i$ levels in ASMC from both WKY and SHR in both normal and Ca^{2+} free buffer. Similar E_{max} values were obtained for either LTD_4 or LTC_4 in both normal and Ca^{2+} free buffer. LTD_4 evoked stronger responses than LTC_4. Most importantly, both LTD_4 and LTC_4 evoked much greater increases in $[Ca^{2+}]_i$ levels in ASMC of SHR than the responses noted in ASMC of WKY strain.

5. In both WKY and SHR, the $CysLT_1$ selective antagonist, MK571, partially blocked LTD_4 but not LTC_4 evoked increases in $[Ca^{2+}]_i$ levels while the dual $CysLT_1/CysLT_2$ receptor antagonist, Bay u9773, abolished the $[Ca^{2+}]_i$ responses to both LTD_4 and LTC_4, suggesting that LTD_4 evoked responses was mediated by activation of both $CysLT_1$ and $CysLT_2$ receptor, while LTC_4 predominantly activate $CysLT_2$ recetopr, resulting in elevated $[Ca^{2+}]_i$ in ASMC.

6. Ang II evoked increases in $[Ca^{2+}]_i$ were markedly attenuated by 2-APB. While CysLT evoked increases in $[Ca^{2+}]_i$ were abolished by 2-APB, suggesting that CysLT recruit predominantly $InsP_3$ sensitive intracellular Ca^{2+} pools in ASMC.

7. Both $CysLT_1$ and $CysLT_2$ receptor mRNA were detected in ASMC from both WKY and SHR. However, the $CysLT_1$ and $CysLT_2$ receptor mRNA levels were much higher in SHR than that in WKY.

Therefore, the CysLT pathway may serve as an additional amplifier pathway in sustaining direct AT_1 mediated Ca^{2+} signaling evoked by Ang II in ASMC. This pathway could be more important in the exaggerated vasoconstrictor responses to Ang II in the SHR.

A schematic diagram elucidating the signal transduction for AT_1 receptor interation with the CysLT system and its role in Ca^{2+} mobilization in NRC and ASMC is presented in

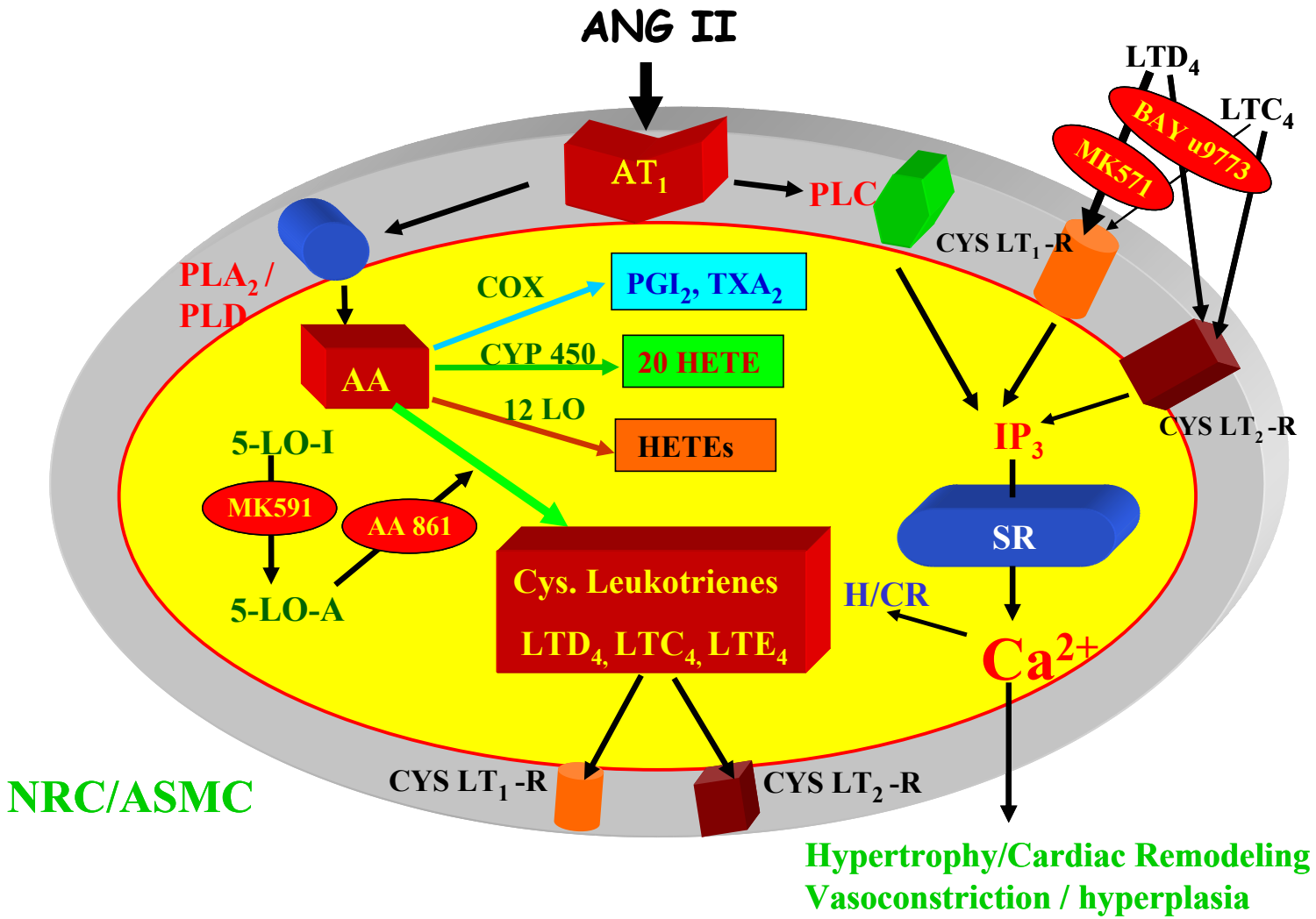


Figure 33. PLA_2/PLD mediated CysLT dependent ancillary signaling in addition to PLC mediated direct Ca^{2+} signaling

7 Future Directions

1. To study the distribution of $CysLT_1/CysLT_2$ specific binding sites on NRC and ASMC. Although $CysLT_1/CysLT_2$ receptor mRNA was detected by northern blot analysis in the present study, radioligand binding studies would provide more detailed characteristics such as the affinity and the site density of the CysLT receptors present in the cardiovascular tissues. This would provide more direct evidence in support of the expression data repoted in the present study.

2. To study the $CysLT_1/CysLT_2$ receptor protein expression in NRC & ASMC. It would be more convincing if the receptor proteins could be directly detected by western blot Analysis.

3. To study the involvement and contribution of CysLT in Ang II evoked cardiovascular hypertrophy and the role of increased $[Ca^{2+}]_i$ in the systhesis of DNA and protein. Although studies have suggested that increase in $[Ca^{2+}]_i$ plays an important role in mediating cardiac and vascular hypertrophy, further study to eluciate the direct effects of CysLT on the systhesis of DNA and protein is important for concluding that CysLT contributes to Ang II evoked cardiovascular hypertrophy.

4. To study the effect of PLA_2 inhibitor on the Ang II evoked increases in Ca^{2+} and CysLT production. PLA_2 is a key enzyme in mediating AA release. Employing a selective PLA_2 inhibitor would provide further supportive evidence for the contribution of CysLT to Ang II evoked Ca^{2+} signaling in both NRC and ASMC.

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